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(54) Title: BIOSENSOR AND METHOD AND INSTRUMENT FOR DEPOSITION OF ALTERNATING MONOMOLECULAR LAYERS

(57) Abstract

A gravimetric biosensor based on two quartz resonators with immobilised layers of antibodies was developed. The active oscillator is covered by antibodies specific to a given antigen, while the reference oscillator is covered by antibodies non-specific to the antigen, in order to compensate the physical absorption effects. In a second aspect the invention provides a method and instrument for depositing films of alternating monomolecular layers, which can contain monolayers of surfactant molecules and monolayers of soluble adsorbed compounds. The first and second aspect in combination can provide a biosensor having alternating monomolecualr layers with at least one layer being biosensitive, e.g. immunosensitive.

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BIOSENSOR AND METHOD AND INSTRUMENT FOR DEPOSITION OF ALTERNATING MONOMOLECULAR LAYERS

The first aspect of the invention relates to biosensors. More particularly, it relates to a transducer comprising two resonators each having a thermally stable layer of protein thereon. Such stability is the subject of British Patent Application No. 9324327.7, WO 95/09058 and reference 4.

Any biosensor includes a sensitive layer and a transducer.

Among different types of transducers for biosensors, the gravimetric one seems to be rather promising as it allows to measure directly mass differences due to the specific attachment of a chemical moiety to the sensitive layer [1]. Low cost and high sensitivity [2] permit to consider it as a promising tool for commercial biosensors.

For example, an immunosensor having an antibody specific for an antigen can be made having the antibody immobilised on the transducer. Several techniques are used for the formation of sensitive antibody layers. Most of them involve chemical immobilisation of antibody molecules on the surface of the transducer. Recently Langmuir-Blodgett (LB) technique began to be applied for the deposition of sensitive layers [3]. The technique enables the formation of dense monomolecular layers at the air/water interface and its transfer onto a solid substrate, providing high density of molecules in the layer and covering homogeneously the surface. The technique does not require a big amount of antibodies and provides good reproducibility of the properties of deposited layers. Moreover, it was shown that dense organisation of proteins

(including antibodies) in the layer by LB technique provides a big improvement in the thermal stability of structure and properties of proteins [4]. This last finding is also very important for immunosensors as it will be possible to keep them without any precaution even in hot places.

The first aspect of the invention will now be described by way of example in more detail with reference to figures 1 to 8 in which:

Figure 1 shows binding curves for 2 different LB samples, namely not heated antibody and heated antibody;

Figure 2 shows a first oscillator for use in air;

Figure 3 shows a second oscillator for use in a liquid;

Figure 4 shows an optocoupling interface;

Figure 5 shows a 24 bit counter;

Figure 6 shows a PC interface board;

Figure 7 shows oscillation frequency variation of an oscillator with successive layers of gold applied thereto; and

Figure 8 shows oscillation frequency variation of an oscillator in contact with water and sucrose solutions of various concentrations.

The aim of the work was to realise and to investigate a gravimetric immunosensor with sensitive layer formed by LB

technique. In order to take into account non specific adsorption, which would cause errors in the measurements, a differential scheme was developed.

A quartz resonator nanobalance provides a valuable immunosensor transducer, as it allows to determine directly the mass changes due to the binding of antigens to antibodies.

A sensor based on two quartz resonators (one active and the second one used as a reference) with immobilised layers of antibodies was developed. The active oscillator is covered by antibodies specific to a given antigen, while the reference oscillator is covered by antibodies non-specific to the antigen, in order to compensate the physical absorption effects.

The electronics is composed of two separate blocks, one designed to acquire by a personal computer the data coming from the other card, which is a 24 bit digital counter directly connected to the two oscillators.

Deposition of antibody monolayers was performed by Langmuir-Blodgett technique with a surface pressure range of 20-35 mN/m onto gluteraldehyde pre-treated quartz resonators.

A thermal treatment of the antibody layer resulted in the reorganisation of the film, and improved the properties of the sensor.

The monolayer of antibodies specific to a given antigen (e.g. specific to insulin) was deposited onto the surface of a quartz resonator while the reference one was covered with a monolayer having no specificity (e.g. an IgG monolayer non-specific to

insulin). The amount of bound antigen was estimated from the difference in frequency shifts according to the Sauerbrey equation [5]:

$$\frac{\Delta f}{f_o} = -\frac{\Delta m}{A\rho\ell}$$

where Δf is the frequency shift, f_o is the initial quartz resonant frequency, Δm is the mass shift, A is the covered area, ρ and ℓ are the quartz density and thickness respectively.

Quartz resonators with the resonant frequency 1-15 MHz, preferably 10 MHz, were used.

A differential scheme has been implemented in order to avoid the measuring problems related to the physical absorption onto the quartz resonators; this undesired contribution to the total signal can affect the measurements rather strongly, and should be carefully eliminated. The proposed system can be divided into three sections. The first block contains the two oscillator circuits and all the high frequency components (and is properly shielded for proper noise rejection); in this block a fast 8 bit counter is used as the first of three cascade counters to form a 24 bit device; 24 bit allow 10 MHz pulses to be counted for more than 1 sec, which ensures a proper resolution in the measurement. The second block latches the parallel outputs of counters into a 24 bit register, and is connected to an interfacing circuitry which provides a suitable connection to a normal personal computer through a standard ISA bus.

The described electronics is controlled by a proper software program, which sends the driving signals to the modules and receives the digital words; the program also implements the differential operations for acquisition.

By way of specific example only, there will now be described the technical design and construction of control equipment for an immunosensor. Careful design is of primary importance for an adequate result, particularly with regard to stability of response.

The immunosensor consists of two quartz oscillators connected to a measuring unit, namely a 24 bit counter. The design of oscillators and the connection with the counter should be optimised in order to measure the incoming pulses at the highest possible resolution.

The design provides a PC controlling card, which can be easily programmed under Microsoft Windows™ or DOS environments. We designed and realised two different types of oscillator, one suitable for measurements in air and the other for measurements in liquids; an appropriate pair of such oscillators is generally used as the system is run in differential mode. However, the possibility is not excluded that under certain circumstances a mixed pair of air/liquid oscillators could be used. The oscillators can work properly in a range of 1 to 15 MHz.

The first oscillator is presented in Figure 2. It utilises a special-purpose IC, namely the HA7210, suitable for oscillators. It is useful for operation in air, as it is very stable; nevertheless it cannot be used in liquids, because it does not provide a sufficient amount of energy to the quartz.

A second scheme is shown in Figure 3. It consists of a TTL integrated circuit directly connected to the quartz. This scheme is useful for measurements in liquid.

One of the most critical blocks in the system is the interface between these oscillators and the measuring unit; in fact a non-ideal interface causes unpredictable results. The solution here implemented is presented in Figure 4. The oscillation signal is fed into an optocoupler (actually two identical systems are visible in Figure 4, due to the possible connection of a reference and a working quartz); each oscillator is intended to be connected to a separate power supply; in addition, a third power supply line is used for the optocouplers; the physical separation of Ground signals of the two oscillators and the counter is important in that it leads to surprisingly stable measurements.

The two signals (square wave outputs of the optocouplers) are then fed into the 24 bit counter by means of a selection logic, presented in Figure 5. Only one of the two signals is sent to the counter at a given time, and the selection is performed by a gate pulse; the gate pulse can either be provided by software (see signals SWEN0 AND SWEN1 of Figure 5) or by hardware (see signals OUT0 and OUT1 of Figure 5).

The device can be easily updated in order to allow use of many quartz oscillators. Referring to Fig. 5, the selection circuit (SWEN0 and SWEN1) can be duplicated or triplicated, to allow more oscillators outputs to selectively reach the counter.

The oscillation signals reach a first 8 bit counter, serially connected to a second one, which is, in turn, connected to the

last one. The parallel outputs of the counters are stored in three respective latches by a LTCH signal coming from the control electronics.

The control electronics is shown in Figure 6; it consists of a card fed into a slot of an ISA BUS of a common Personal Computer (IBM or compatible). The control electronics provides the Chip Select signals (see CSxxxx) for the various devices, and performs Input and Output operations to/from the PC board.

The user can program this card in order to provide digital signals to control the counter; one example is the software gate pulse already described. A second possibility to get the gate pulse is by programming a programmable counter/timer (see 8253 of Figure 6), which provides the above mentioned OUTx signals.

In order to check the described electronics, a quartz oscillator was covered by a layer of gold of different thicknesses, by successive evaporations; this increases the mass on the oscillator and as a consequence a progressive oscillation frequency decrease is expected. The result is visible in Figure 7.

A check of the system in liquids has also been performed; the resulting frequency decrease at different densities and viscosities of a liquid in contact with one side of the quartz has been investigated. The result is shown in Figure 8, which relates to solution of water and sucrose at different concentrations.

Insulin antibodies from Immunological group of Bakh Institute, Russian Academy of Sciences were used in the study. Rabbit and

mouse antibodies were used for evaluating non specific binding. Insulin was purchased from Sigma Co., and working solutions were prepared at a concentration of 0.5 mg/ml.

Monolayers can be prepared in LB trough (MDT, Russia) according to the method described in [3]. Films can be transferred onto quartz and silicon substrates by horizontal lift technique at 25 mN/m surface pressure.

The measuring procedure was the following; resonators each covered by a sensitive layer (respectively antigen specific or antigen non-specific monolayers) were placed in the antigen solution for increasing periods of time; after the reaction, the resonators were washed in order to remove non-specifically adsorbed antigen and measurements of the frequency shift were carried out after drying with nitrogen. However, the possibility is not excluded that under certain conditions the measurements of frequency shift could be carried out with the resonator still in the liquid phase (refer to Figure 3 above).

The system can be equipped with an array of flow chambers, so providing multiple parameter acquisition in liquid, for example different compositions and/or concentrations can be used in different solutions.

Heating of the samples has been carried out in a normal oven at 150°C for 30 minutes.

X-ray measurements were done by small-angle X-ray diffractometer with linear position-sensitive detector, providing an angular resolution of 0.02° [6].

Surface potential measurements were carried out by a home-made device using vibrating electrodes (Kelvin probe) [7].

In Fig. 1, antibody-antigen reaction kinetics are shown with symbols as follows: (+) is experimental data points of not heated antibody layer; (x) is experimental data points of antibody layer after heating; solid line is the best fit of (+) data; dashed line is the best fit of (x) data.

The most striking result is connected with the behaviour of the heated sample. First of all, antibody activity appears preserved in LB film [3].

Second, the antibody-antigen reaction kinetics show that, after thermal treatment up to 423 K, the saturation level (reached when all the available binding sites are filled with antigens) was increased by 25% and a 6-fold increase in the reaction rate occurred.

As these phenomena could very unlikely be due to modifications of the single antibody molecule activity, as induced by temperature, only a modification in the film organisation can be responsible for them.

To explain the increased plateau level, therefore, we thought that a large amount of specific binding sites became available in the reaction with insulin.

In fact, it was shown [7] that the heating process provides enough energy to allow film recrystallisation after coming back to room temperature. Lipid [7] and protein LB films showed a better order after such heating procedure. In our case, due to

the weak amphyphilic properties of antibodies, there is a certain statistical distribution in the orientation of IgG molecules at the air/water interface and, therefore, after deposition not all the binding sites are exposed to antigen solution; moreover, there is the possibility of flip-flop transitions for molecules in deposited LB films [8]. Heat-induced recrystallisation could provide a more regular distribution of binding sites, namely, more F_{ab} fragments could be oriented towards the antigen solution.

A higher amount of molecules oriented in the same direction on the solid substrate means that the monolayer becomes more anisotropic. In this case, charges and dipole distribution inside each antibody should provide an effective electric field in the direction normal to the film plane. In order to test this idea, surface potential measurements on solid substrates were performed. These measurements have shown to be sensitive to the charge and dipole distribution in the layer. Comparison between the surface potential of a not heated film and that of a heated sample is reported in Table I. The marked increase in the surface potential confirms the previous statement and helps in giving an insight into the phenomenon of the reaction rate variation. This electric field can be the source of the additional driving force which causes this 6-fold increase. In fact, the appearance of an electric field can easily explain the kinetic variation through electrostatic interactions between antibodies and antigens, as it was already shown for the interaction of the same IgG with other organic molecules [9].

Table I

	surface potential [mV]
1 monolayer just after deposition	-84.5
after heating at 423K for 30 mins	-370

To prove directly that the recrystallisation process takes place also in antibody LB films, X-ray diffraction measurements were carried out. A just deposited film does not display any order in the direction normal to the film plane. Instead, in case of heated samples, a Bragg reflection appears in the X-ray pattern. The angular position of the maximum corresponds to a periodicity of 115 Å, clearly pointing out a strong improvement in the order of the film. The spacing value, in fact, corresponds to the length of IgG molecules.

Summarising the results, it was shown that the combination of differential gravimetric transducer with LB sensitive layer exposed to thermal treatment is suitable to reach better results for immunosensor fabrication.

This aspect of the invention has been described with reference to an immunosensor. However, it will be apparent to those skilled in the art that a nanogravimetric balance biosensor may be used to determine directly a mass change due to other types of specific or non-specific binding to a protein monolayer on a quartz resonator. Examples considered to be within the scope of the invention include but are not limited to:

a) selective capture of DNA and RNA molecules for the purpose

of DNA and RNA analysis.

b) specific detection of pesticides in solutions.

The second aspect of the invention provides a method and an instrument for depositing the films built up from monomolecular layers of several types alternating in a required sequence, which can contain the monolayers of surfactant molecules and the monolayers of soluble adsorbed compounds.

Organic films produced by successive transfer of monomolecular layers onto the solid substrates find applications, in particular, in electronics for creation of super thin high sensitive photoresist and electron beam resist coverings, dielectric layers for the metal-dielectric-semiconductor structures of precisely determined and controlled thickness and composition, as well as analysing "crystals" for long-wavelength X-Ray irradiation, sensors for ions, gases, and biosensors such as immunosensors.

Illustrative material on the prior art and the present invention is shown in the Figures 9 to 16.

Figure 9 shows a classical method of LB film deposition and one possible example of a complex structure realised with the use of two separated troughs;

Figure 10 shows an example of the structure of alternating monolayers and process of its deposition with a known KSV System 5000;

Figure 11 shows instrument for deposition of alternating

monolayers according to the present invention.

Figures 12 and 13 show the method of the invention;

Figure 14 shows an instrument for realising the method presented in Figures 12 and 13;

Figure 15 shows a simple example of the structure which can be deposited with the instrument shown in Figure 14; and

Figure 16 shows a particular design of an instrument for deposition of alternating monolayers including adsorbed monolayers.

Figure 17 shows a block diagram of the electronics/software of the instrument.

Langmuir-Blodgett (LB) technique is one of the well-known methods of film deposition by successive transferring of monomolecular layers of surfactant compounds onto the solid substrates (Fig.9). To produce the film by this method one spreads the solution of amphyphilic molecules in organic solvent at the surface of aqueous subphase (1) which is poured into the trough (2). As a rule, molecules consist of hydrophilic groups interacting strongly with water and long hydrocarbon chains. After evaporation of the solvent the monolayer is compressed with barrier (3) (Fig.9(a)(b)) up to definite surface pressure which is measured with balance (4). Solid substrate (5) is moved successively down and up through the air-water interface. Monolayers are deposited onto the substrate as it is shown in the Figs. 9(c)(d). Feedback system (6) provides the required surface pressure value during the process of film deposition.

The molecules of the last deposited monolayer are practically always arranged with inert hydrophobic tails in the direction of the air medium after pulling out the substrate from aqueous subphase. When somebody tries to remove the monolayer from the air-water interface after immersing the substrate into aqueous subphase and then to pull it out through clean surface, the last deposited monolayer is transferred back onto the air-water interface or recrystallizes onto the substrate resulting in hydrophobic surface of the latter. Molecules of very few types only can be directed with hydrophilic groups to the air medium. Immersion of the substrate with the film after completion of the process shown in Fig.9(d) into some solution for adsorption of the dissolved compound onto hydrophilic groups of surfactant molecules will not give any result because active hydrophilic groups are protected with inert hydrophobic layer. Designs of usual LB instruments with one compartment enable one to deposit the films consisting of monolayers of one type. If two separate troughs or one trough with two separate compartments are used one can deposit the structure of alternating bilayers of different molecules similar to that shown in Fig.9(e), but deposition of alternating monolayers (Fig.10(a)) is impossible.

To realize the structure of alternating monolayers, in which hydrophilic surfaces of different monolayers are in contact, several variants of the instruments are proposed. Commercial LB instrument KSV System 5000 (Figs.10(b)-(f)) comprises the trough (2) divided at the level of air-water interface into two compartments (7a,7b). To deposit the film of alternating monolayers the substrate (5) is immersed into aqueous subphase through the monolayer of the first type in the compartment 7a (Figs.10(b),(c)), is transferred to the compartment 7b under the water by means of rotation of the holder (8)(Fig.10(d),(e)), and

is pulled out from the aqueous subphase through the monolayer of the second type (Fig.10(f)). Different subphases can not be used for forming different monolayers at the air-water interface in such instruments because transfer of substrate under water from the first compartment to the second one is necessary. The films of alternating monolayers deposited with similar instruments after pulling out the substrates from aqueous subphase practically always terminate with inert layers of hydrophobic tails of the molecules as in the case of usual LB multilayers (e.g. Fig.9(e) and Fig.10(a)).

Some embodiments of the instruments for deposition of the LB films are described in
Thin Solid Films, 134 (1985)83;
Thin Solid Films, 133(1985) 235;
Molecular Engineering with the KSV 5000 LB System, KSV Inc., Helsinki (1989).

Patent application EP 0 119 126 A1 describes a method and several embodiments of an instrument design for depositing the films of alternating monolayers, which are considered as the prototypes of method and instrument provided by the present invention. The method enables one to realize the contact between hydrophilic surfaces of neighbouring monomolecular layers of different types in the process of LB film deposition. As in the case of KSV System 5000 mentioned above the goal is achieved due to the possibility to transfer the substrate from the first compartment of the trough to the second one under the water. Several variants of the instrument are proposed which give the possibility to accomplish such a transfer. The embodiments using translational motion of the holder with substrate to pass the latter from first compartment to the

second one are the closest to the present invention (Fig.11). Special shutter (9) divides the trough (2) of the instrument into two compartments (7a,7b) at the level of air-water interface. The monolayers of two different types are spread at the surfaces of water subphase (1) in the first and in the second compartments respectively. They are compressed with independent barriers (3a,3b), and the required values of surface pressure for each monolayer is maintained during film deposition. The shutter (9) prevents the monolayers from the two compartments mixing with each other, but makes it possible to transfer the holder (8) with the substrate (5) from the first compartment to another when the substrate is immersed into aqueous subphase. Several versions of shutter realization are described. When the substrate is immersed into subphase in the first compartment (7a) through the first monolayer the latter is deposited in such a manner that its hydrophilic surface is in contact with water as in the case shown above in the Fig.1(c). For this reason, when the substrate is pulled out from subphase in other compartment (7b) after passing through the shutter (9) monolayer of the second type is deposited by hydrophilic surface onto the hydrophilic one of the first monolayer. Repetition of this procedure results in the deposition of the film consisting of alternating monolayers of different types. As in the case of KSV System 5000 all embodiments of the prior art instruments require the use of common subphase for formation of the monolayers at the air-water interface of various compartments.

The methods proposed before and designs of the existing instruments for deposition of the LB films including method and designs of the instruments provided by EP 0 119 126 A1 do not provide the possibility to pull out the substrate from aqueous

subphase with the layer of hydrophilic groups located at the external boundary of the film. That restricts the possibilities of the instruments on the use of different subphases for formation of the monolayers of different types at the air-water interface as well as on the use of adsorption of soluble compounds from the solutions onto hydrophilic groups of the surfactant molecules.

The present invention provides the method and the instruments for depositing the monolayers of different types alternating in the required sequence onto the solid substrate which allow
- to transfer a monomolecular layer of a first type from the surface of aqueous subphase of definite composition and temperature and a monomolecular layer of the second type over the first one from the surface of aqueous subphase of another composition and temperature so that hydrophilic surfaces of these different monolayers are in contact with each other;
- to accomplish during the film formation an adsorption of soluble compounds from solutions of different compositions and temperatures onto hydrophilic surfaces of the deposited monolayers of the surfactant compounds.

The goal is achieved due to providing the possibility to pull out the substrate from aqueous subphase so that the hydrophilic surface of the deposited monolayer appears to be the external boundary of the film. Such situation is possible only in the case when the last monolayer is in contact with the polar liquid, i.e. with the aqueous solution in the present example. The proposed solution of the problem is shown schematically in the Figs.12(a)-(e). To provide contact of the monolayer with aqueous solution after pulling out the substrate from subphase 1a a mobile plate (10) covers the immersed substrate (5)

creating a gap (11) between the plate and the substrate, which is able to hold aqueous solution inside due to capillary forces when the substrate together with the covering plate are transferred to the air medium. Then the deposition of new monomolecular layer (Figs.12(f)-(j) or adsorption of some dissolved compound (Figs.12(k)-(p)) can be carried out. To deposit the monolayer of another type the substrate (5) together with covering plate (10) and aqueous solution in the gap (11) is first transferred to the second separate compartment (7b) of the trough (2) containing subphase (1b) of the required composition and temperature as well as the compressed monolayer at the air-water interface (Fig.12(f)). Then the substrate and the plate are immersed together in the subphase 1b of the compartment 7b (Fig.12(g)). Then the plate is moved relatively to the substrate uncovering the latter (Fig.12(h)), and the substrate is transferred from the subphase through the monolayer so that the deposition takes place (Fig.12(i)). Resulting structure is shown in the Fig.12(j). To carry out adsorption of the soluble compound onto hydrophilic surface of th monolayer the substrate (5) covered by plate (10) with aqueous solution in the gap (11) is transferred to the separate compartment (12a) containing the solution (13a) of the compound at the required temperature (Fig.12(k)). The substrate and the plate are immersed together in the solution (Fig.12(l)). The plate is moved relatively to the substrate uncovering the latter for the time interval necessary for adsorption of the compound (Fig.12(m)). Then the plate again covers the substrate (Fig.12(n)), and the substrate together with covering plate and with the solution in the gap is transferred from the compartment 12a into the air medium (Fig.12(o)). Resulting structure is shown in the Fig.12(p). Then the created structure can be transferred to any other compartment for deposition of

monomolecular layers (7a, 7b) or for adsorption of another compound (12b). To avoid contamination of the solutions used in the different compartments by the solution contained in the gap between the plate and the substrate as well as to remove surplus of non-specifically adsorbed compound (see Fig.12(p)) one or several compartments (12c, for example) can be used for washing of the sample. Washing of the sample can be carried out in some solution or in pure water as shown in the Fig. 13. The sequence of operations is the same as that in the case of adsorption. The result of washing of the sample obtained after adsorption (Fig.12(p)) is shown in the Fig.13(f).

The instrument (Fig.14(a)) making it possible to realize the method of deposition of alternating monolayers shown in the Figs.12,13 comprises

- the trough (2) for deposition of the monolayers of surfactant compounds consisting of several completely separated compartments (7a,7b,7c,....);
- separated compartments for adsorption of the soluble compounds onto the hydrophilic surfaces of the monolayers or for washing of the samples (12a,12b,12c,....);
- independent barriers (3a,3b,3c.....) compressing the monolayers at the air-water interface in every compartment of the trough;
- balances (4a,4b,4c,.....) measuring surface pressure in every compartment of the trough;
- feedback systems (6a,6b,6c,....) maintaining required values of surface pressure in every compartment of the trough;
- system (14) for movement of the substrate (5) in the air medium between different compartments (7a,7b,....12a,12b,...), for dipping the substrate through the surface of subphase of the selected compartment, and for pulling it out from the subphase;
- mobile plate (10) covering the substrate (5) to create a gap

(11) (Figs.14(b),(c) between the plate and the substrate capable to hold the solution inside it due to capillary forces.
If the deposition of alternating monolayers onto both surfaces of the substrate is necessary the instrument contains two mobile plates (10a and 10b in the Fig.14(d)) capable to cover and uncover each surface of the substrate.

A simple example of the structure which can be deposited with this instrument is shown in Fig.15.

In the particular embodiment of the instrument shown in Fig.16 trough (2) consists of two compartments for deposition of the monolayers of surfactant compounds (7a,7b). Two balances (4a,4b) measure the surface pressure in these compartments of the trough. Feedback systems are not shown. Two compartments (12a,12b) are used for adsorption of soluble compounds onto hydrophilic surfaces of the monolayers. One compartment (12c) is used for washing of the samples. Trough and compartments for adsorption and washing are placed in a thermostat (15) to control temperature in every section. Motors 16a and 16b move the barriers (3a,3b). System (14 in the Fig.14(a)) of movement of the substrate (5) comprises
- two synchronously operating motors (17a,17b) moving up and down vertical bars (18a,18b);
-two horizontal bars (19a,19b) attached to the vertical ones;
- mobile block (20) with the holder (8) of substrate moving along the horizontal bars by motor (21) installed onto the block (20) itself;
-mobile plate (10) and motor (22) installed onto the block (20) moving the plate 10 relatively to the substrate.
Installation of the motor (21) onto the mobile block (20) enables one to use non-limited number of compartments in the

instrument although five compartments only are used in the instrument shown in the Fig.16.

Carrying out the procedures and operating the instrument present in the Fig.16 for producing the structure shown in the Fig.15, where A and B are monolayers of different surfactant molecules and C is the adsorbed monolayer of soluble compound, occurs as follows:

-Compartment 7a of the trough (2) is filled with aqueous solution suitable for depositing monolayers of type A. Compartment 7b of the trough is filled with aqueous solution suitable for depositing monolayers of type B. Compartment 12a is filled with aqueous solution of the compound C, which can be specifically adsorbed onto hydrophilic surface of monolayer B under conditions created in the compartment. Compartment 12b is not used. Slow flow of distilled water is created in the compartment 12c. Flows of water of the required temperatures are created in the appropriate sections of thermostat to ensure optimal conditions for the processes of deposition and adsorption.

-Hydrophobic substrate 5 is put in the holder 8.

-Solution of surfactant compound A in the organic solvent is spread at the air-water interface of compartment 7a. Solution of surfactant compound B in organic solvent is spread at the air-water interface of compartment 7b. After evaporation of the solvents the monolayers are compressed with the barriers 3a and 3b up to the surface pressures necessary for deposition. Balances 4a and 4b measure the values of surface pressure. Feedback systems connecting balances 4a and 4b with motors 16a and 16b respectively maintain the values of surface pressure at a constant level during operation of the instrument.

-Mobile block 20 with substrate 5 in holder 8 is moved by motor

21 along the bars 19a and 19b to the compartment 7a of the trough. The substrate is disposed above the surface of aqueous subphase.

-Construction comprising vertical bars 18a and 18b, horizontal bars 19a and 19b, and mobile block 20 is moved by motors 17a and 17b down immersing the substrate into aqueous subphase through the monolayer A. Then the substrate 5 moves up and again down in the same manner. Three monolayers of type A appear to be deposited onto the substrate.

-Mobile plate 10 is moved down by motor 22 relatively to the substrate 5 covering the latter.

-Construction comprising vertical bars 18a and 18b, horizontal bars 19a and 19b, and mobile block 20 is moved up by motors 17a and 17b pulling out the substrate 5 with plate 10 and aqueous solution in the gap between their surfaces from the subphase.

-Mobile block 20 with the substrate 5 covered by plate 10 is moved by motor 21 to compartment 7b.

-Construction comprising vertical bars 18a and 18b, horizontal bars 19a and 19b, and mobile block 20 is moved down by motors 17a and 17b immersing the substrate 5 covered by plate 10 in aqueous subphase of compartment 7b.

-Mobile plate 10 is moved up by motor 22 relatively to the substrate 5 uncovering the latter.

-Construction comprising vertical bars 18a and 18b, horizontal bars 19a and 19b, and mobile block 20 is moved up by motors 17a and 17b pulling out the substrate 5 from aqueous subphase through the monolayer B. Monolayer of B type is deposited over the last monolayer of A type so that hydrophilic surfaces of the monolayers A and B are in contact. Then the substrate 5 moved down through the monolayer B in the same manner. The next monolayer of B type is deposited and it hydrophilic surface is the external boundary of the film.

-Mobile plate 10 is moved down by motor 22 relatively to the substrate 5 covering the latter.

-Construction comprising vertical bars 18a and 18b, horizontal bars 19a and 19a, and mobile block 20 is moved up by motors 17a and 17b pulling out the substrate 5 with plate 10 and aqueous solution in the gap between their surfaces from the subphase.

-Mobile block 20 with the substrate 5 covered by plate 10 is moved by motor 21 to the compartment 12a.

-Construction comprising vertical bars 18a and 18b, horizontal bars 19a and 19b, and mobile block 20 is moved down by motors 17a and 17b immersing the substrate 5 covered by the plate 10 in aqueous solution of compartment 12a.

-Mobile plate 10 is moved up by motor 22 relatively to the substrate 5 uncovering the latter for the time interval necessary for adsorption of the compound C onto hydrophilic surface of the monolayer B.

-Mobile plate 10 is moved down by motor 22 relatively to the substrate 5 covering the latter.

-Construction comprising vertical bars 18a and 18b, horizontal bars 19a and 19b, and mobile block 20 is moved up by motors 17a and 17b pulling out the substrate 5 with plate 10 and aqueous solution in the gap between their surfaces from the compartment 12a.

-Mobile block 20 with the substrate 5 covered by plate 10 is moved by motor 21 to the compartment 12c.

-Construction comprising vertical bars 18a and 18b, horizontal bars 19a and 19g, and mobile block 20 is moved down by motors 17a and 17b immersing the substrate 5 covered by the plate 10 in the distilled water of compartment 12c.

-Mobile plate 10 is moved up by motor 22 relatively to the substrate 5 uncovering the latter for the time interval necessary for dissolution of non-selectively adsorbed surplus

of the compound C.

-Mobile plate 10 is moved down by motor 22 relatively to the substrate 5 covering the latter.

-Construction comprising vertical bars 18a and 18b, horizontal bars 19a and 19b, and mobile block 20 is moved up by motors 17a and 17b pulling out the substrate 5 with plate 10 and distilled water in the gap between their surfaces from the compartment 12c.

-Mobile block 20 with the substrate 5 covered by plate 10 is moved by motor 21 to the compartment 7a.

-Construction comprising vertical bars 18a and 18b, horizontal bars 19a and 19b, and mobile block 20 is moved down by motors 17a and 17b immersing the substrate 5 covered by the plate 10 in aqueous subphase of compartment 7a.

-Mobile plate 10 is moved up by motor 22 relatively to the substrate 5 uncovering the latter.

-Construction comprising vertical bars 18a and 18b, horizontal bars 19a and 19b, and mobile block 20 is moved up by motors 17a and 17b pulling out the substrate 5 from aqueous subphase through the monolayer A. Monolayer of A type is deposited over the adsorbed monolayer C.

It will be readily apparent that the technique of the second aspect of the invention can be applied to the first aspect so as to form quartz resonators for use in a biosensor. Particularly a biosensor may be produced where a biosensitive monolayer (e.g. a protein monolayer such as an antibody monolayer) may be formed as a second or outer layer deposited upon a first or inner layer (such as of surfactant).

Electronics of the Trough

The electronic design of the trough must take into account precision positioning of the moving parts. Even if mechanical equipment must be very stable and with reproducible motion, electronics must not affect this overall precision.

Moreover, mainly for barrier driving purposes, the control electronics must be able to perform a feedback operation able to maintain the surface pressure at a preset value with high accuracy; this detail is very important to obtain good quality films, as the surface pressure when depositing layers plays a key role in the process; in general, the more the surface pressure is constant, the better the resulting films.

On the other hand, the same barriers should be quickly moved when not depositing, in order to minimise the time needed for the preparation of a new deposition; this point is important during barriers repositioning or during system reset; in both cases the barriers must be moved at the trough edges before the next deposition session.

The electronics should also measure different signals useful to monitor the actual deposition in process, such as the surface pressure or the temperature; about this point, a rather high resolution must be ensured in order to perform the necessary operations following each reading (feedback control). One can roughly estimate to design and utilise 12 bit AD and DA converters, properly interfaced to a Personal Computer. The acquisition rate is not critical, and the time between acquisitions from different sensors depends upon the feedback algorithms used; for instance we intend to utilise a PID

(Proportional Integral Derivative) procedure for the barriers positioning while maintaining a constant surface pressure.

Another very important consideration is about the noise produced by the rotating stepper motors; taking into account this problem means avoiding loss in resolution due to vibrations; the latter can also cause oscillations in the system.

A good but critical method to avoid vibrations due to the motors is the division of the unit step into fractions of step, while the motor is rotating; dividing a step into multisteps can be accomplished by creating a stable state between the motor coils and the axis at intermediate positions within a unit step; basically such driving implies the creation of square waves at different duty cycle values, and the consequent driving of the motor with these waveforms. This solution allows a smooth and regular rotation of the motor, in contrast with the usual sharp steplike motion; different multistep divisions can be obtained, ranging from 1/2 step to 1/128 step.

The driving software must be at least of two different types: very low level routines to drive the electronics, and very high level programs to present a user friendly interface. It is strictly important to have the low level software directly interfacing with the hardware, in order to provide a quick and reliable system driving. On the other hand, it is suitable to allow the user to deposit films without many troubles and in the easiest way; the user should be provided with simple instructions to drive the system. A block diagram of the electronics/software system is shown in Figure 17.

The following considerations are not actually present in

commercially available products.

The driving system of the barriers (electronics plus mechanics) can reliably provide horizontal motion steps of 1 μm. The motors driving system implements multistep logic to create smooth rotations, to diminish the noise and to eliminate vibrations. The software package allows the user to easily write programs for deposition protocols, by utilising an "ad hoc" language dedicated to the system; the programs are automatically compiled and checked for their consistency and correctness; then deposition process automatically starts following the directives of the actual protocol.

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Claims

1. A quartz resonator nanogravimetric balance capable of acting as a biosensor to determine directly a mass change by measurement of a differential resonator frequency shift, which comprises a transducer having a first active quartz resonator and a second reference quartz resonator, each resonator having immobilised thereon a biosensitive monolayer, and wherein said monolayer on said first resonator comprises molecules specifically sensitive to a given chemical moiety and said monolayer on said second resonator comprises molecules non-specifically sensitive to said given moiety; whereby said mass change is due to binding of said moiety to said molecules.

2. A biosensor according to claim 1, wherein said biosensitive monolayer is a protein monolayer.

3. A quartz resonator nanogravimetric balance according to claim 1, capable of acting as an immunosensor to determine directly a mass change due to the binding of antigen to antibody by measurement of a differential resonator frequency shift, which comprises a transducer having a first active quartz resonator and a second reference quartz resonator, each resonator having immobilised thereon an immunosensitive protein monolayer, and wherein said monolayer on said first resonator comprises an antibody specific to a given antigen and said monolayer on said second resonator comprises an antibody non-specific to said given antigen.

4. An immunosensor according to claim 3, wherein deposition of antibody monolayers is performed by the Langmuir-Blodgett technique preferably with a surface pressure in the range of 20-

35 mN/m.

5. An immunosensor according to claim 3, wherein deposition of antibody monolayers is performed onto gluteraldehyde pre-treated quartz resonators.

6. An immunosensor according to claim 3, wherein after being deposited at least one antibody layer is thermally stabilised by heating the resonator and returning it to room temperature after predetermined time resulting in reorganisation, recrystallisation and increased density of packing of the antibody protein.

7. An immunosensor according to claim 3, wherein said heating is to a temperature of about 423K for about 30 minutes.

8. An immunosensor according to claim 3, wherein said first monolayer antibody is insulin specific and said second monolayer antibody is rabbit or mouse IgG.

9. An immunosensor according to claim 3, wherein the quartz resonators have a resonant frequency between 1 and 15 MHz, preferably about 10 MHz.

10. An immunosensor substantially as hereinbefore described with reference to Figures 1 to 8 of the drawings.

11. Use of an immunosensor according to any of claims 3 to 10 to determine a mass change due to binding of antigen to antibody, which comprises the following steps:

i) measuring an original quartz resonant frequency (f_o) for

said antibody-covered resonators of said transducers;

ii) placing said resonators in a solution of said given antigen for a predetermined period of time to allow a binding reaction to occur;

iii) removing said resonators from said solution and washing to remove non-specifically adsorbed antigen;

iv) drying said resonators;

v) measuring a frequency shift Δf; and

vi) calculating an amount of bound antigen Δm according to the following equation:

$$\frac{\Delta f}{f_o} = -\frac{\Delta m}{A\rho\ell}$$

wherein A is the covered area and ρ and ℓ are the quartz density and thickness respectively.

12. A biosensor according to claim 1, wherein at least one resonator has immobilised thereon alternating monomolecular layers produced by the method of any of claims 16 to 20.

13. Control and detection means for a nanogravimetric balance biosensor according to claim 1, comprising means for supplying power to each oscillator, means for counting oscillations received from an oscillation in a predetermined period of time,

means for selectively coupling each oscillator in turn to the counting means, and means for determining a frequency shift caused by binding of antigen to antibody on one of the resonators.

14. Control and detection means according to claim 13, wherein each oscillator has a separate ground connection.

15. Control and detection means according to claim 13 or 14 provided on a card for insertion into a personal computer.

16. Method of deposition of alternating monomolecular layers, wherein a solid substrate (5) is immersed in a liquid subphase in a first compartment (7a) of a trough (2) having the conditions for depositing monomolecular layers of a first type,
- is transferred into a second compartment (7b) of the trough (2) having the conditions for depositing monomolecular layers of a second type,
-is pulled out from liquid subphase of said second compartment (7b),
-and the process of immersion of the substrate into liquid subphase and pulling said substrate out from the latter in different compartments of the trough is reiterated;

for the purpose of increasing the number of types of molecules capable of inclusion into the system of alternating monolayers as well as for providing the possibility of more extensive variation of the conditions of deposition

said method comprising:

covering the surface of the substrate (5) immersed in liquid subphase (1a) of the first compartment (7a) chosen for deposition of the monolayer of the first type with mobile plate (10) creating a gap (11) between surfaces of said plate and

substrate capable to hold liquid inside due to capillary forces;
-pulling out the substrate (5) covered by mobile plate (10) from the subphase (1a) of the first compartment (7a) together with liquid inside the gap (11);
-transferring the substrate (5) covered by mobile plate (10) to the second compartment (7b) having the conditions for forming the monolayer of second type different from the first one on the substrate;
-immersing the substrate (5) covered by mobile plate (10) into liquid subphase (1b) of said compartment;
-moving the mobile plate (10) relatively to substrate (5) uncovering the surface of the latter; and
-forming the monolayer of the said second type on the substrate.

17. Method according to claim 16, for forming the monolayer of the said second type from surfactant molecules

said method comprising

pulling out the substrate (5) uncovered by the plate (10) from the liquid subphase (1b) of said second compartment (7b) through the surfactant monolayer situated at the surface of the subphase.

18. Method according to claim 16, for forming the monolayer of said second type due to adsorption of molecules from the solution onto hydrophilic surface of said first type monolayer

said method comprising:

keeping the substrate (5) uncovered by the plate (10) and immersed in solution (13a,13b,....) of the chosen compartment (12a,12b,....) for the time interval necessary for adsorption of dissolved molecules onto the hydrophilic surface of the previously deposited monolayer;

- moving the mobile plat (10) relatively to substrate (5)

covering the latter and creating the gap (11) capable to hold solution inside between the surfaces of said plate and substrate due to capillary forces;

- pulling out the substrate (5) covered by mobile plate (10) with solution inside said gap from the solution (13a,13b,....) of said compartment (12a,12b,....); and

- carrying out the process of deposition of another monolayer of surfactant compound, or the process of adsorption of another soluble compound, or the process of removal of non-specifically adsorbed molecules.

19. Method according to claim 18, for removing molecules not specifically adsorbed during said process of adsorption of dissolved compound from the solution (13a,13b,....) of the chosen compartment (12a,12b,....) onto the hydrophilic surface of the monolayer

said method comprising:

transferring the substrate (5) covered by mobile plate (10) with the solution of said adsorbed compound inside the gap (11) to the compartment (12a, 12b,....) containing solution (13a,13b,....) without said adsorbed compound;

- immersing the substrate (5) covered by plate (10) into the solution of said compartment;

- moving the plate (10) relatively to the substrate (5) uncovering the latter for the time interval necessary for removal of non-specifically adsorbed compound;

- moving the mobile plate (10) relatively to substrate (5) covering the latter and creating the gap (11) capable to hold solution inside between the surfaces of said plate and substrate due to capillary forces; and

- pulling out the substrate (5) covered by mobile plate (10) with solution inside said gap from the solution (13a,13b,....)

of said compartment (12a,12b,.....).

20. Method according to claim 16, for forming a monolayer on a solid substrate

said method comprising:

carrying out the processes of deposition of surfactant compounds, adsorption of dissolved molecules onto hydrophilic surfaces of monolayers, and removal of non-specifically adsorbed compound from the substrate in separate compartments (7a,7b,....12a,12b,....) capable of containing liquid solutions of different compositions and temperatures.

21. An instrument for deposition of alternating monomolecular layers by means of immersion of a solid substrate into a liquid subphase of a first compartment of a trough having the conditions for depositing monomolecular layers of a first type, transferring said substrate into a second compartment of the trough having the conditions for depositing monomolecular layers of a second type, and pulling out the substrate from the liquid subphase of said second compartment; said instrument comprising

a trough (2) for deposition of monolayers separated into a number of compartments (7a,7b,....),

- barriers (3a, 3b,....) compressing the monolayers of surfactant compounds at the surfaces of liquid subphases in said compartments,
- balances (4a,4b,.....) measuring surface pressure in every compartment of the trough (2),
- a system (14) for movement of the substrate (5) between said compartments, for dipping the substrate into the liquid subphase of every compartment, and for pulling out said substrate from said liquid subphase,
- a thermostat (15) for control of the temperature of liquid

subphases in every compartment;

for the purpose of increasing the number of types of the molecules capable for inclusion into the system of alternating monolayers as well as for providing the possibility of more extensive variation of the conditions of deposition by means of creation of possibility for pulling out the substrate from the liquid subphase together with layer of liquid contacting with hydrophilic surface of monolayer and for transferring said substrate with said layer of liquid into the liquid subphase of another composition;

said instrument further comprising:

a mobile plate (10) capable of covering the substrate (5) and creating a gap (11) between surfaces of said plate and said substrate holding liquid solution inside due to capillary forces.

22. An instrument according to claim 21, for the purpose of inclusion in the monolayers of soluble compounds into the system of alternating monolayers;

said instrument comprising:

compartments (12a,12b,....) for adsorption of soluble compounds onto hydrophilic surfaces of the monolayers.

23. An instrument according to claim 21, for providing additional possibilities for variation of the conditions of deposition;

said instrument comprising:

separate compartments (7a,7b,.....12a,12b,....) capable of containing liquid subphases of different compositions and temperatures.

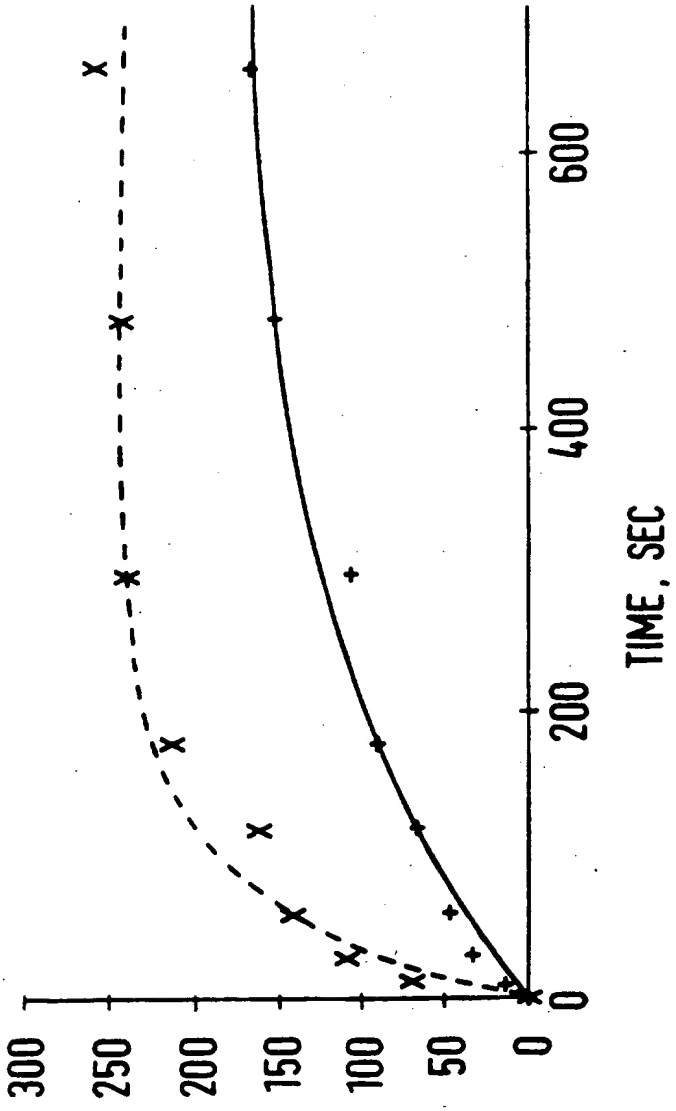
24. An instrument according to claim 21, for the purpose of

using any number of compartments (7a,7b,....12a,12b,....) for formation of the monolayers on the substrate;

said instrument comprising:

a motor (21) attached to the holder (8) of the substrate (5) capable of moving the substrate (5) and mobile plate (10) together from the first compartment to any other compartment.

FIG. 1



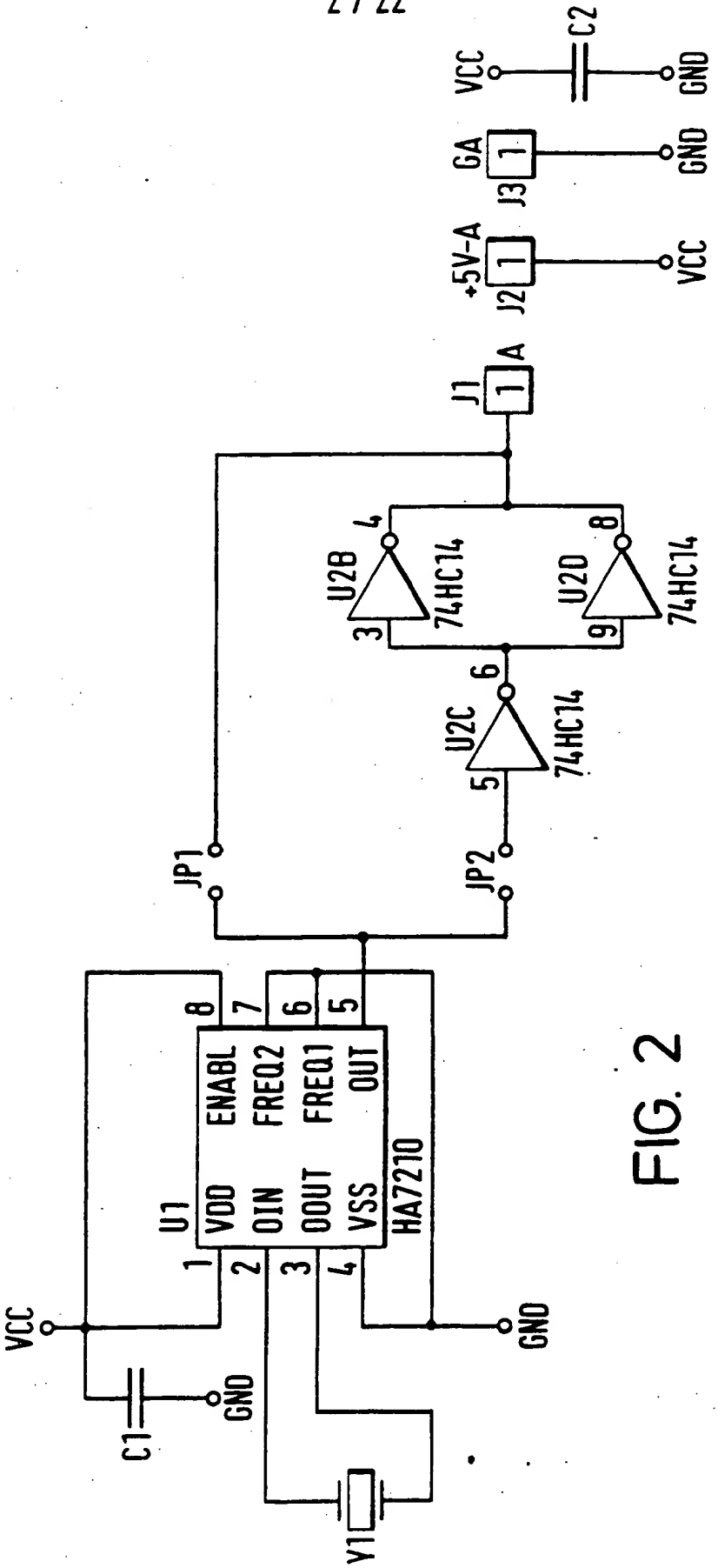

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FIG. 2

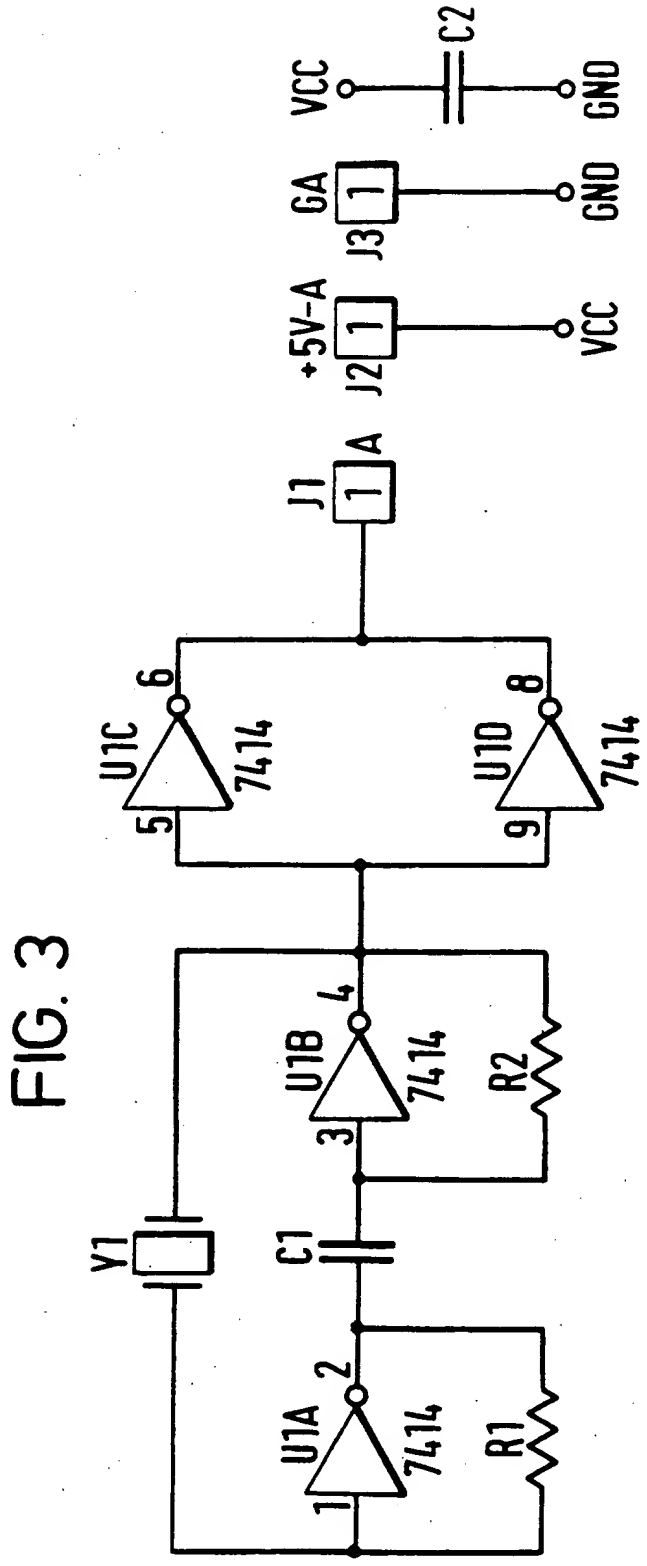
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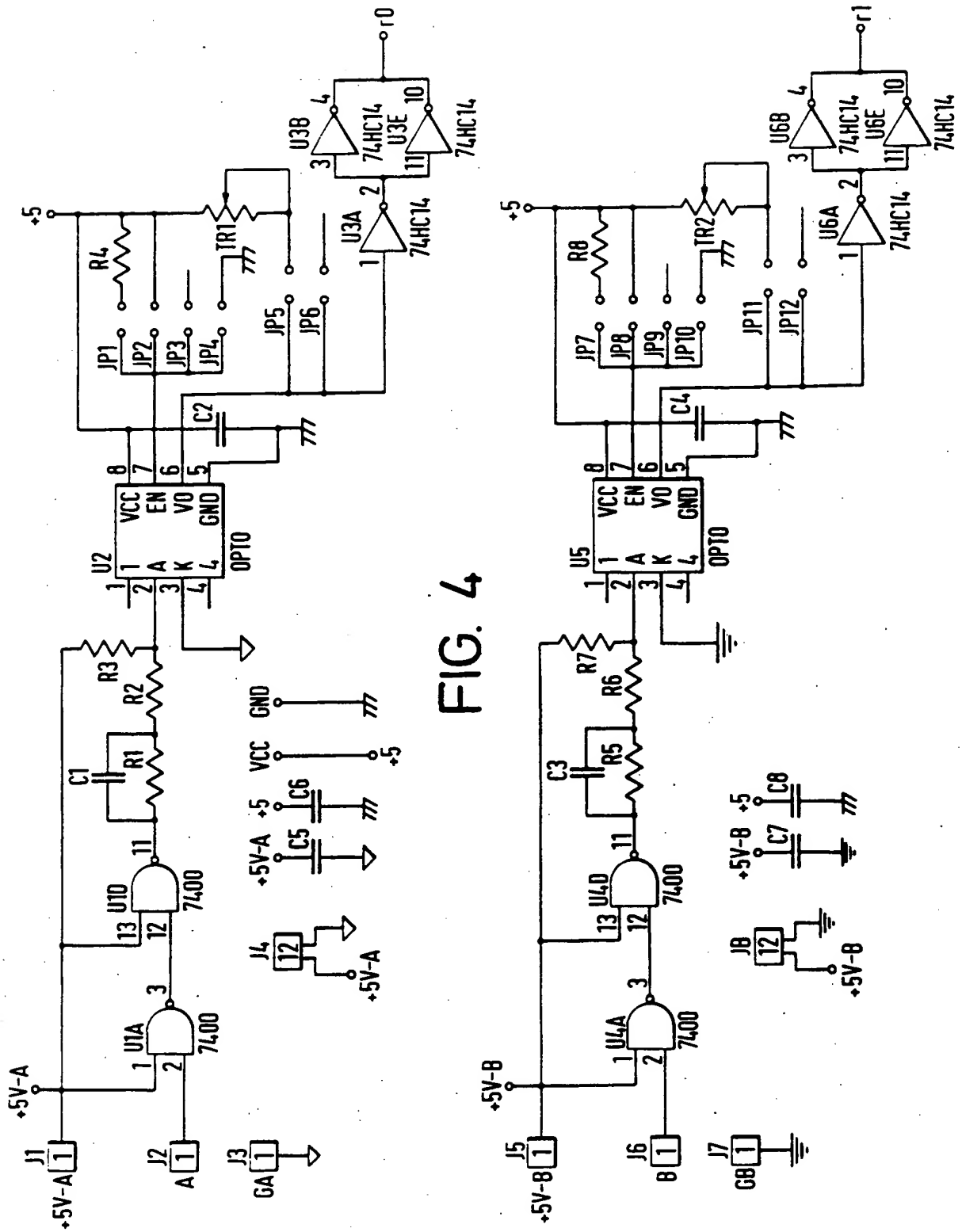
FIG. 3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J6
J7
J8
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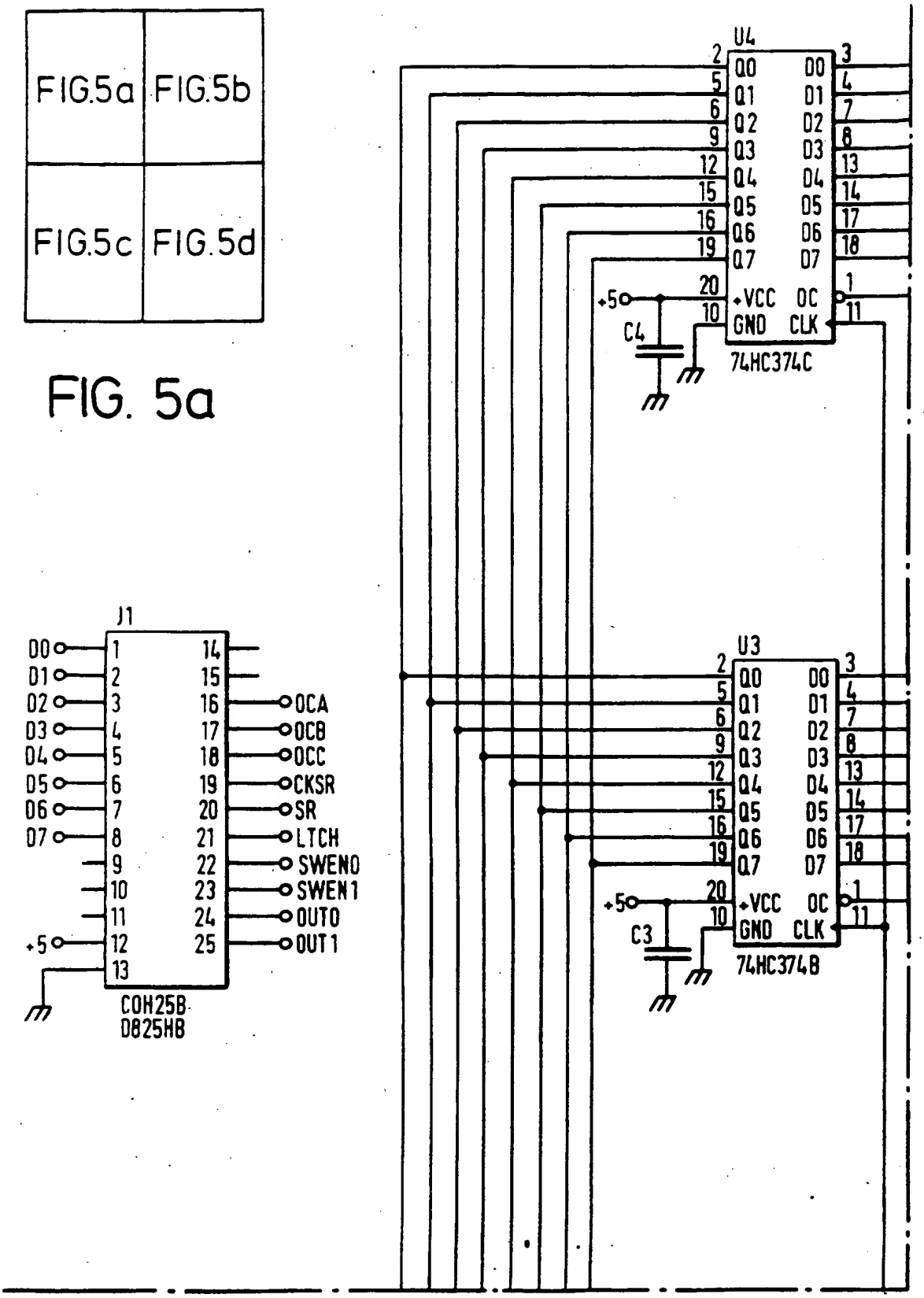
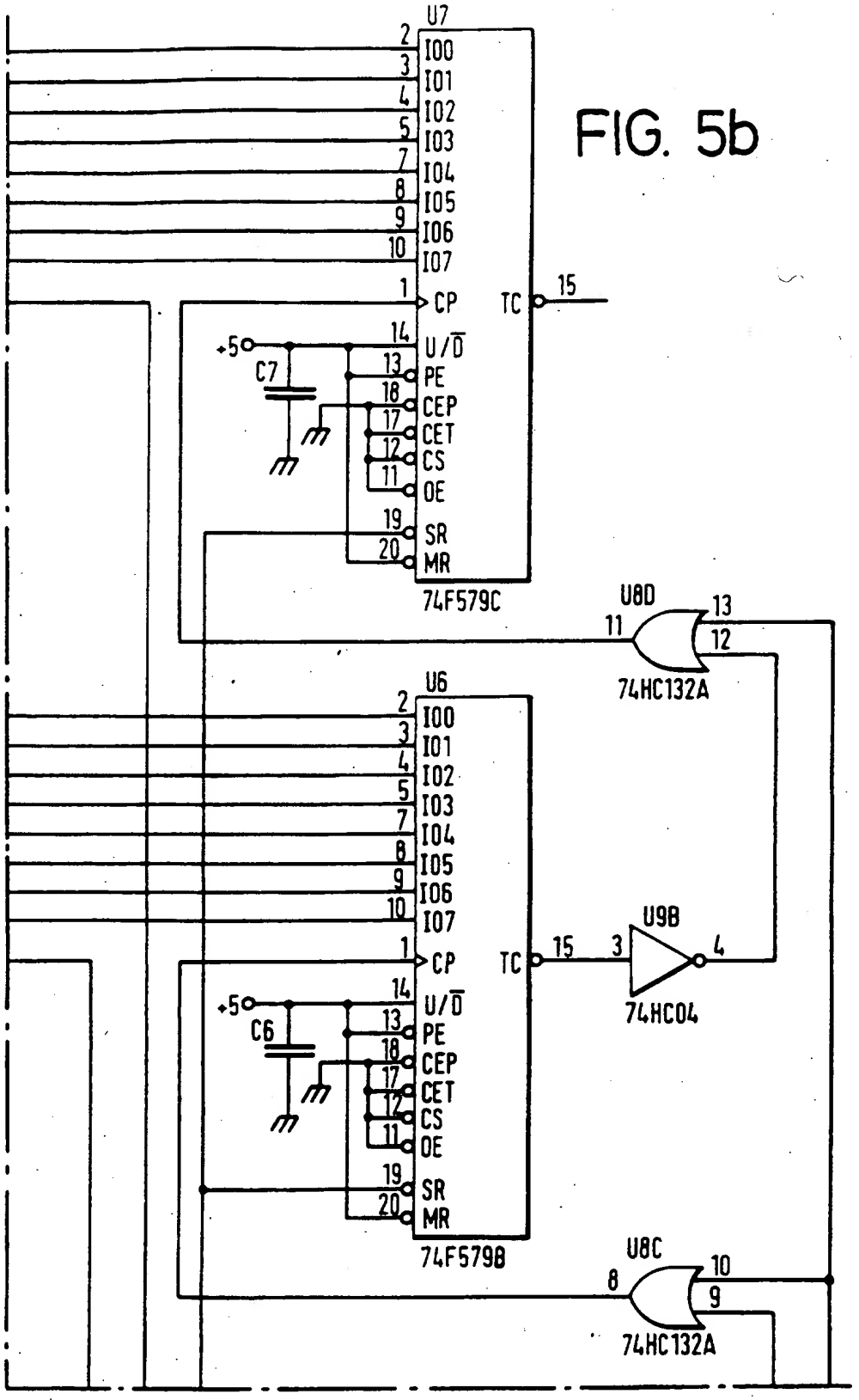
r1

FIG. 4

FIG.5a	FIG.5b
FIG.5c	FIG.5d

FIG. 5a

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8 IO5
9 IO6
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13 PE
18 CEP
17 CET
12 CS
11 OE
19 SR
20 MR
74F579C
U8D
11
13
12
74HC132A
U6
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3 IO1
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5 IO3
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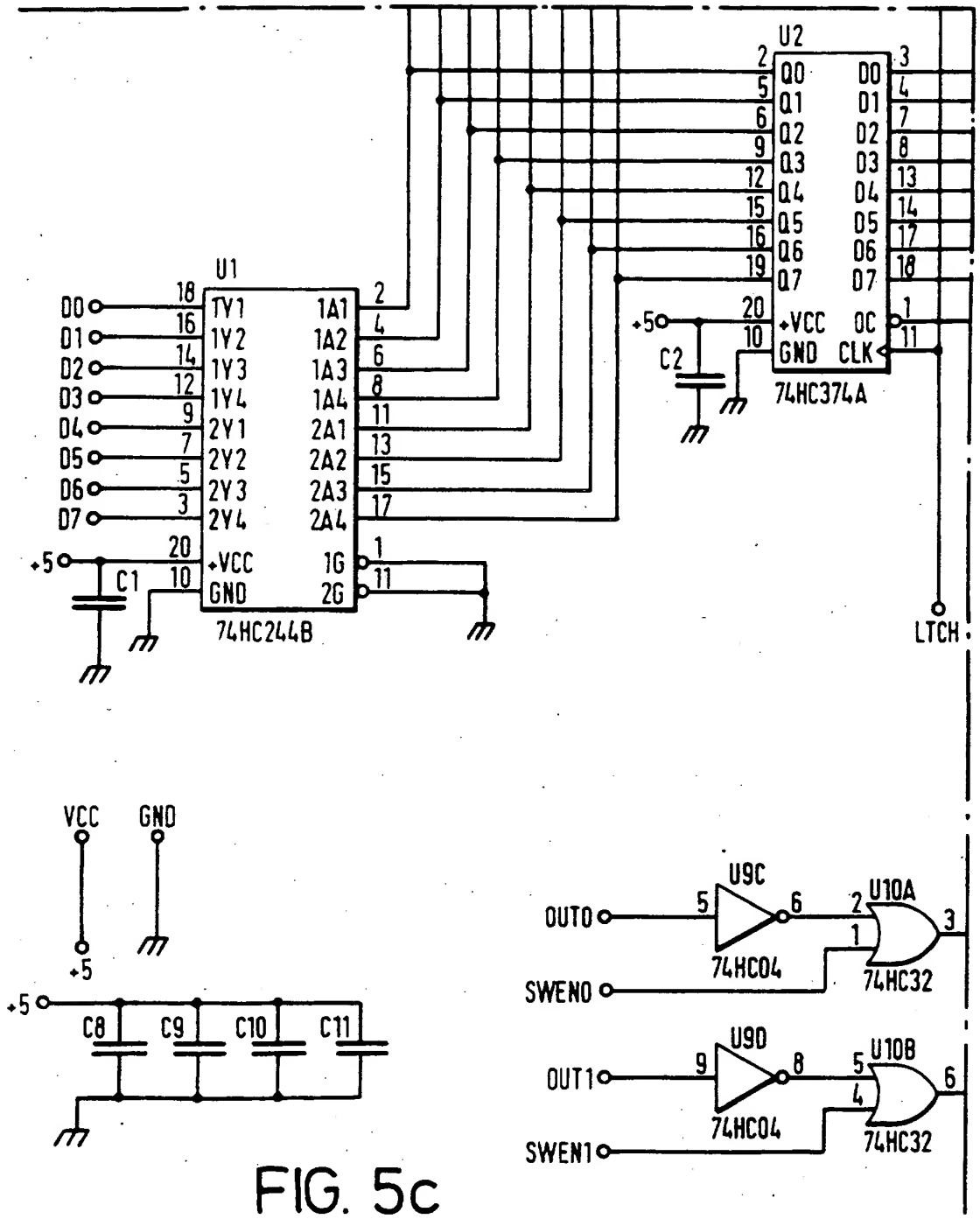
FIG. 5b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D6
D7
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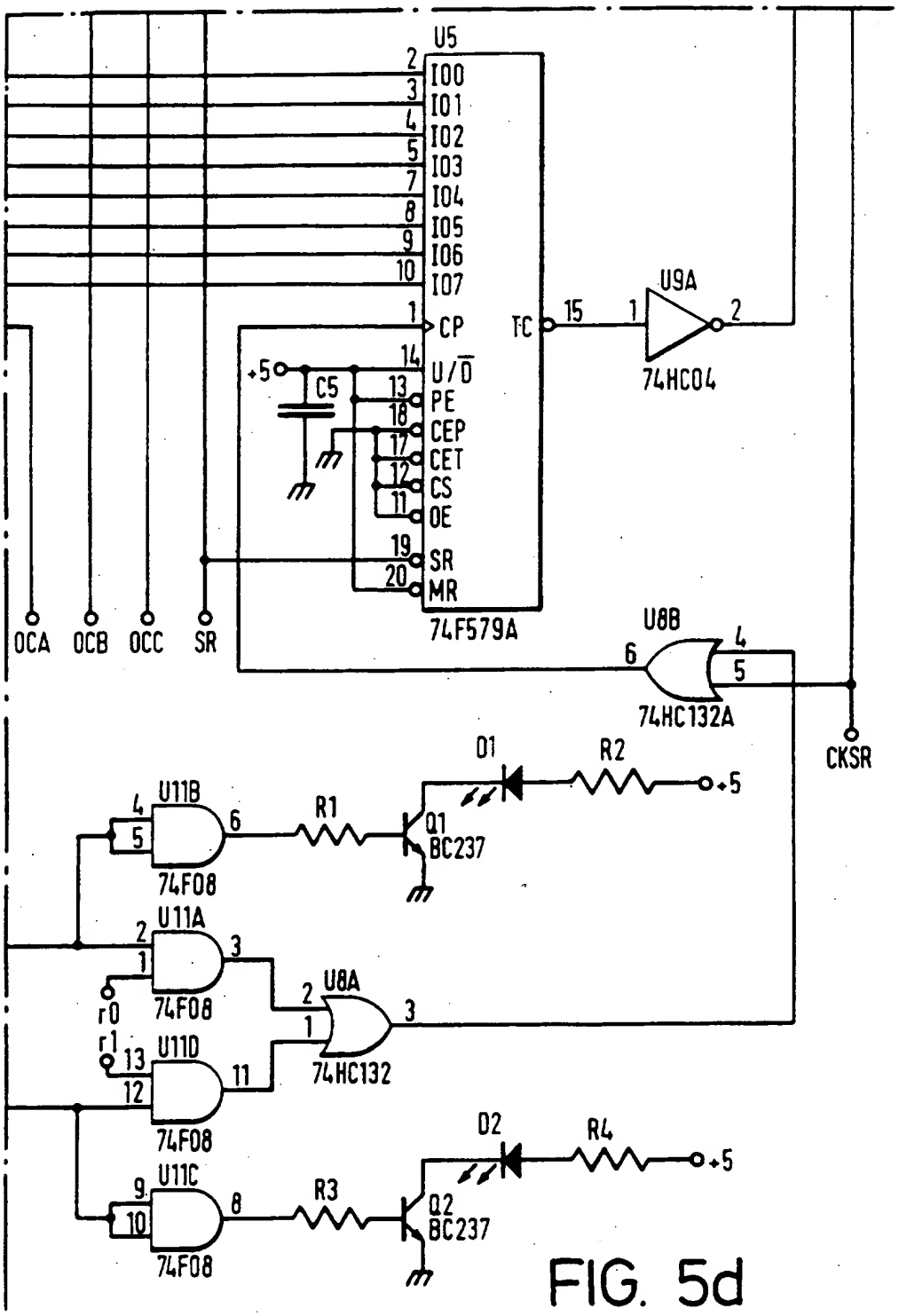
FIG. 5c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C5
OCA
OCB
OCC
SR
U8B
74HC132A
CKSR
D1
R2
U11B
74F08
R1
Q1
BC237
U11A
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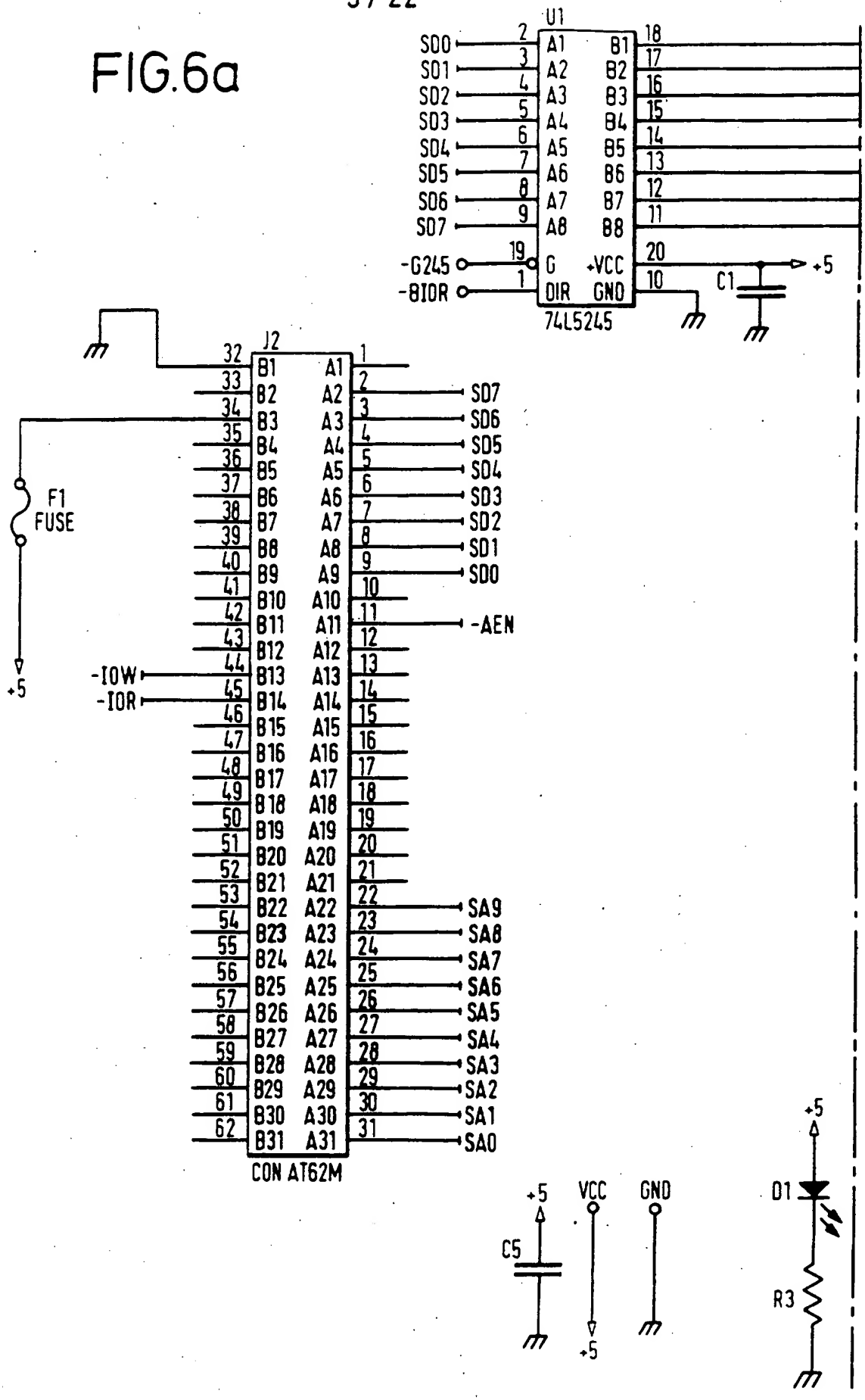
Q2
BC237

FIG. 5d

FIG.6a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B3
B4
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B7
B8
G
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GND
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J2
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FUSE
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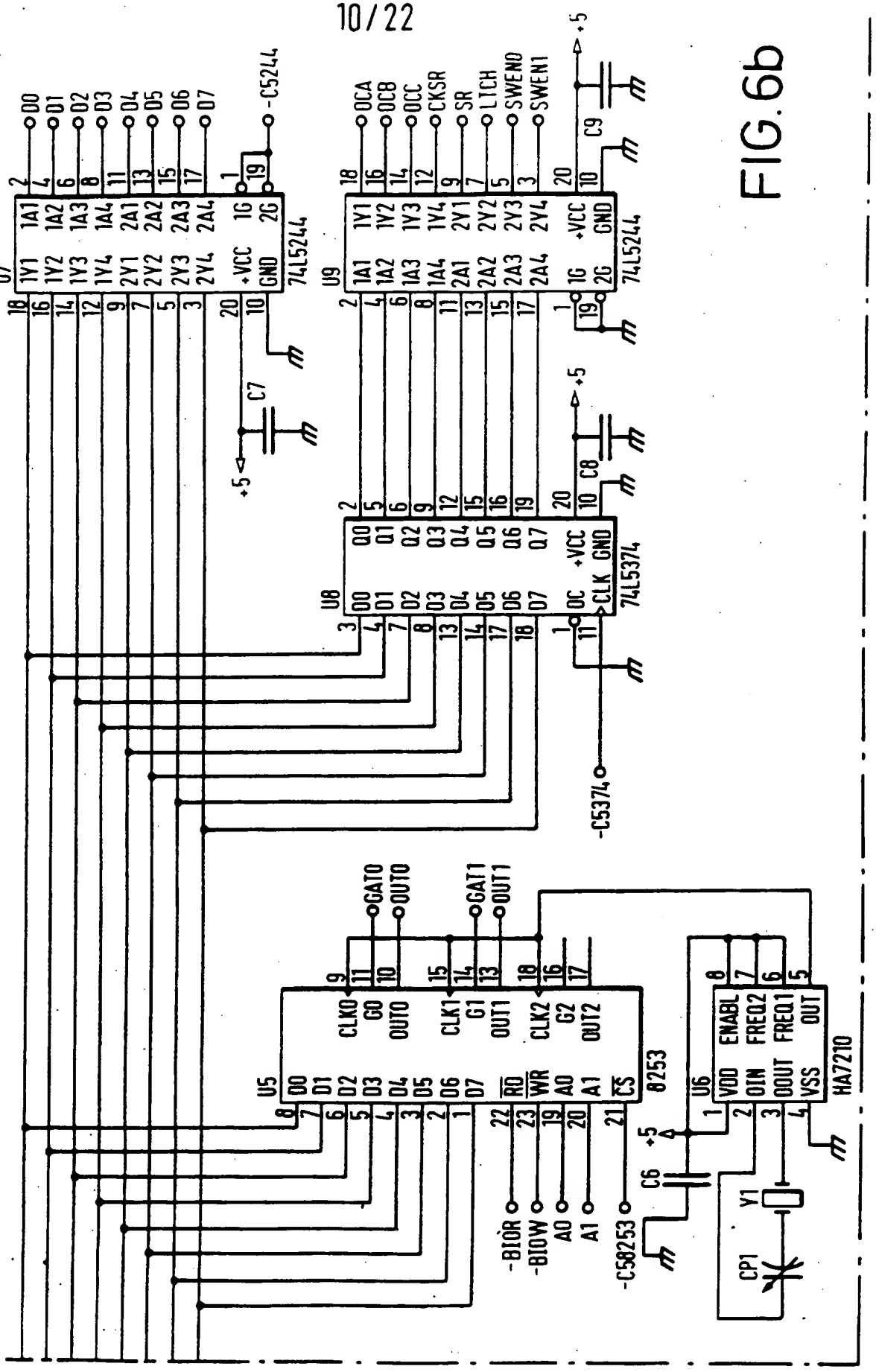
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D4
D5
D6
D7
OCA
OCB
OCC
CKSR
SR
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SWEN0
SWEN1
+5
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-C8253
-BIOR
-BIOW
A0
A1
C6
C7
C8
C9
Y1
CP1
GAT0
OUT0
GAT1
OUT1
CLK0
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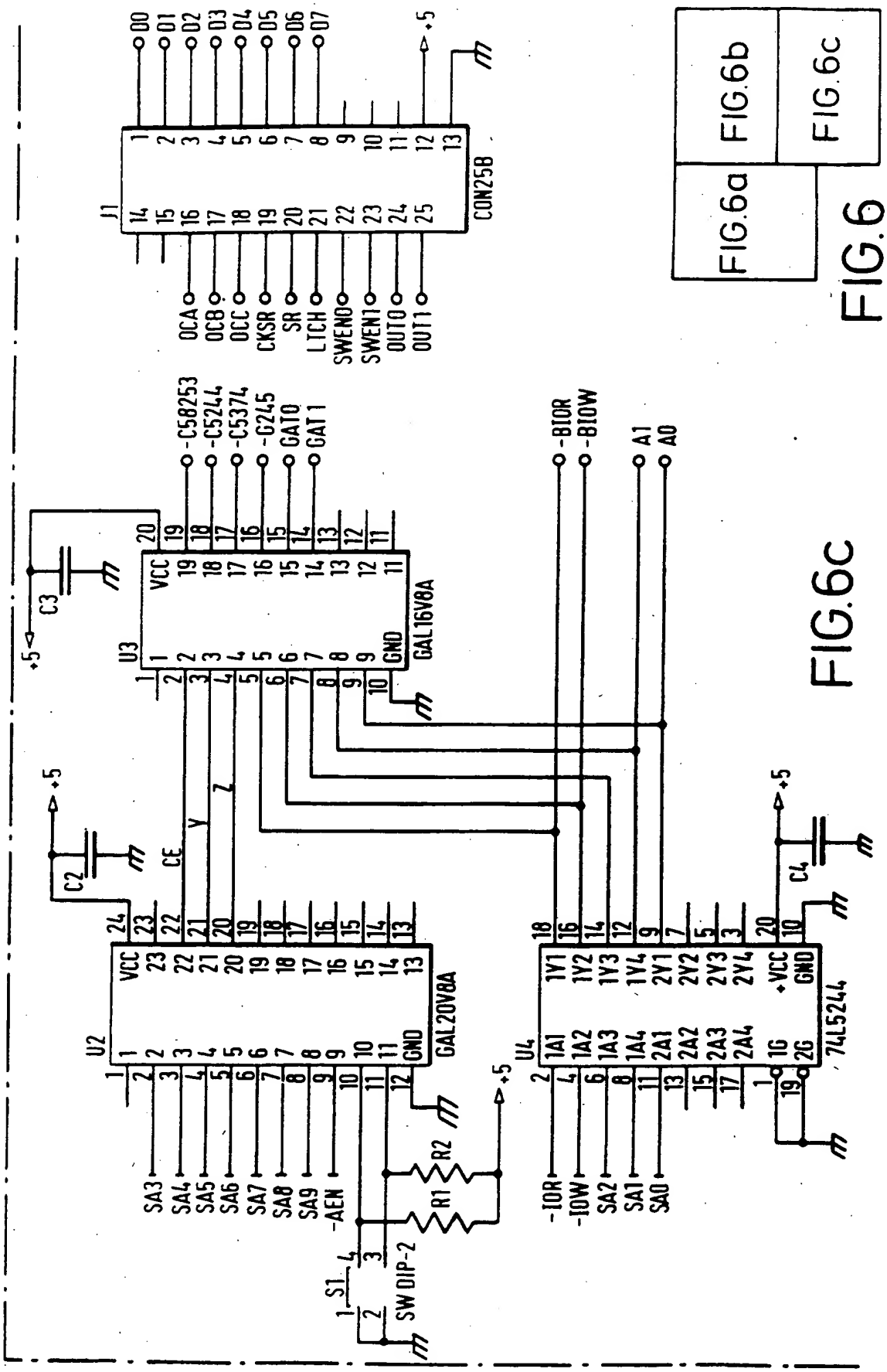
G1
OUT1
CLK2
G2
OUT2
RD
WR
CS
ENABL
FREQ2
FREQ1
OUT
VDD
OIN
OOUT
VSS

FIG.6b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-BIOR
-BIOW
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A0
C3
U3
VCC
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C2
CE
Y
Z
U2
GAL20V8A
C4
U4
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1Y2
1Y3
1Y4
2Y1
2Y2
2Y3
2Y4
+VCC
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SA4
SA5
SA6
SA7
SA8
SA9
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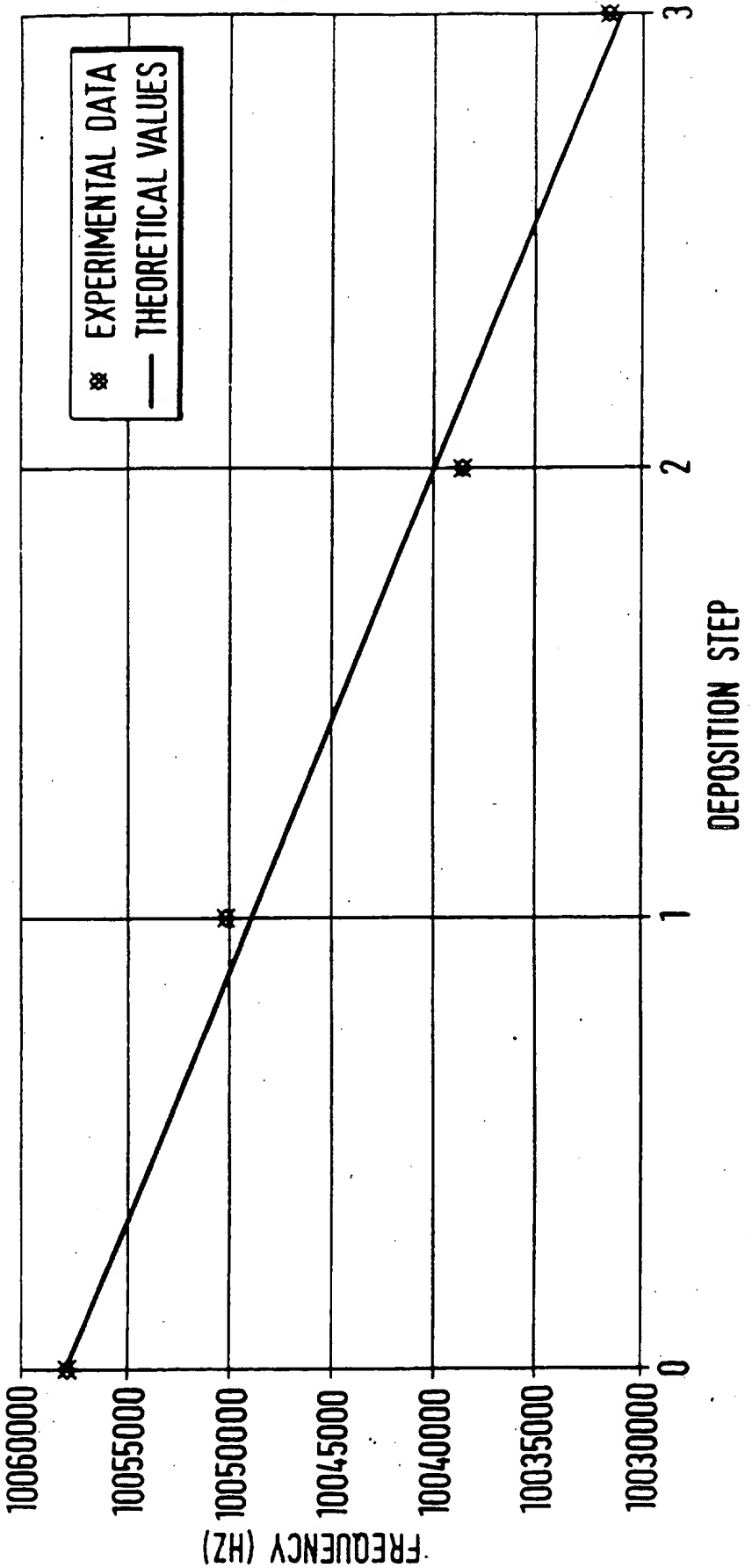
R2
R1
S1
SW DIP-2
-IOR
-IOW
SA2
SA1
SA0

FIG.6

FIG.6a
FIG.6b
FIG.6c

FIG.6c

FIG. 7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2
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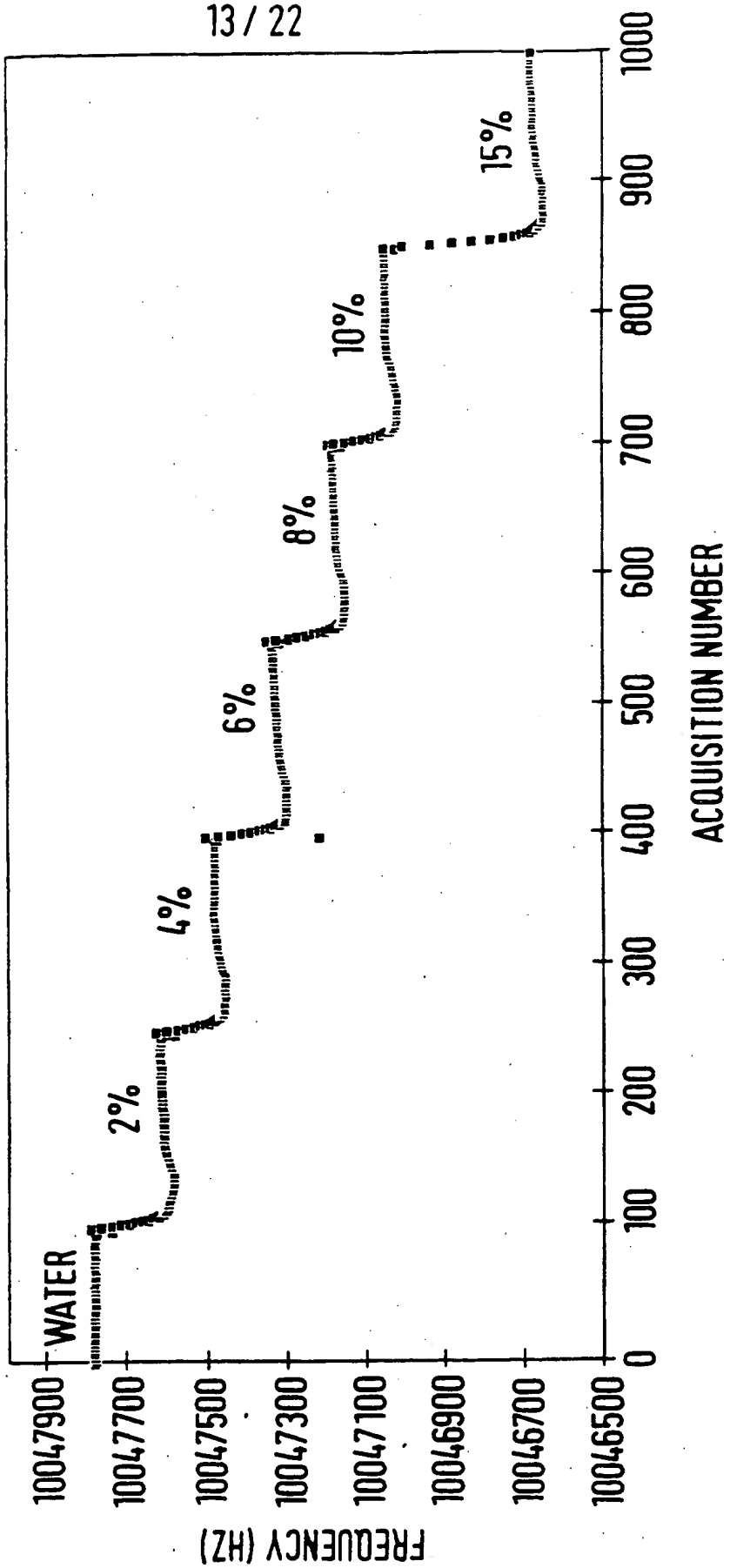
FIG. 8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4%
6%
8%
10%
15%

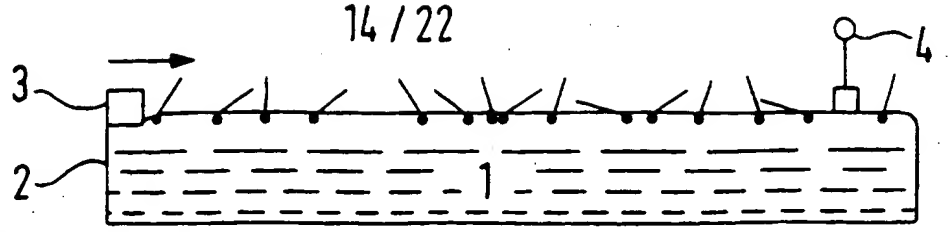


FIG. 9(a)

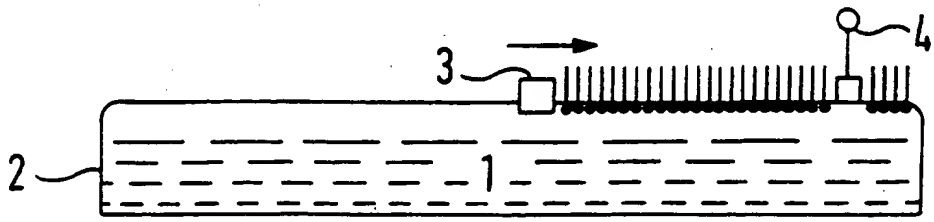


FIG. 9(b)

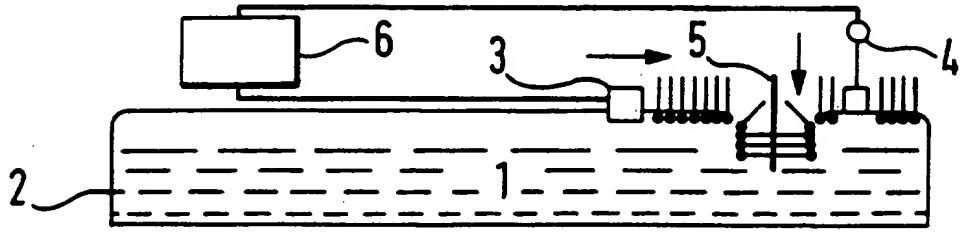


FIG. 9(c)

FIG. 9(d)

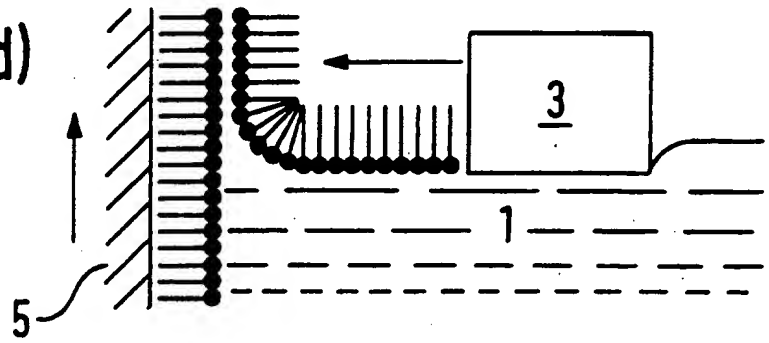
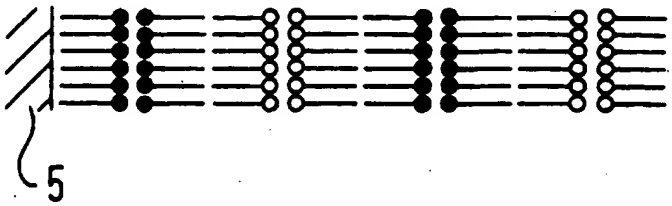


FIG. 9(e)



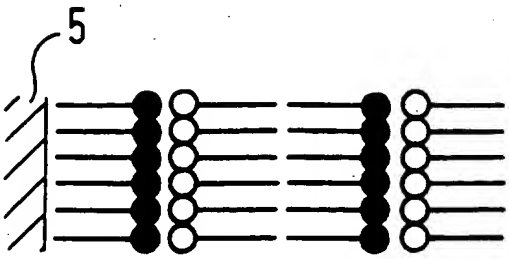


FIG. 10(a)

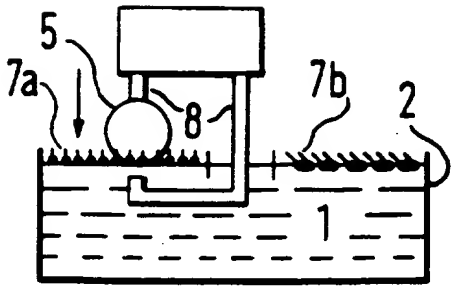


FIG. 10(b)

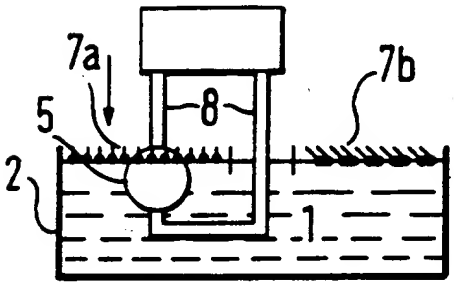


FIG. 10(c)

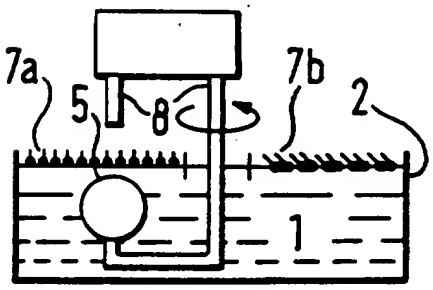


FIG. 10(d)

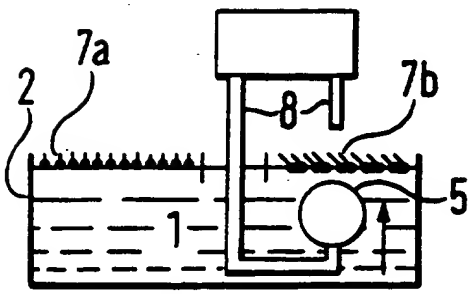


FIG. 10(e)

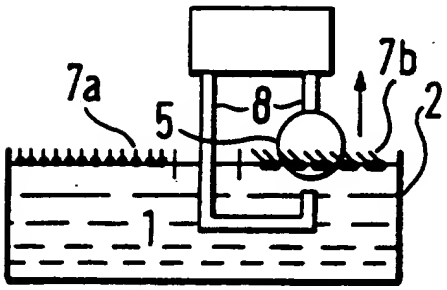
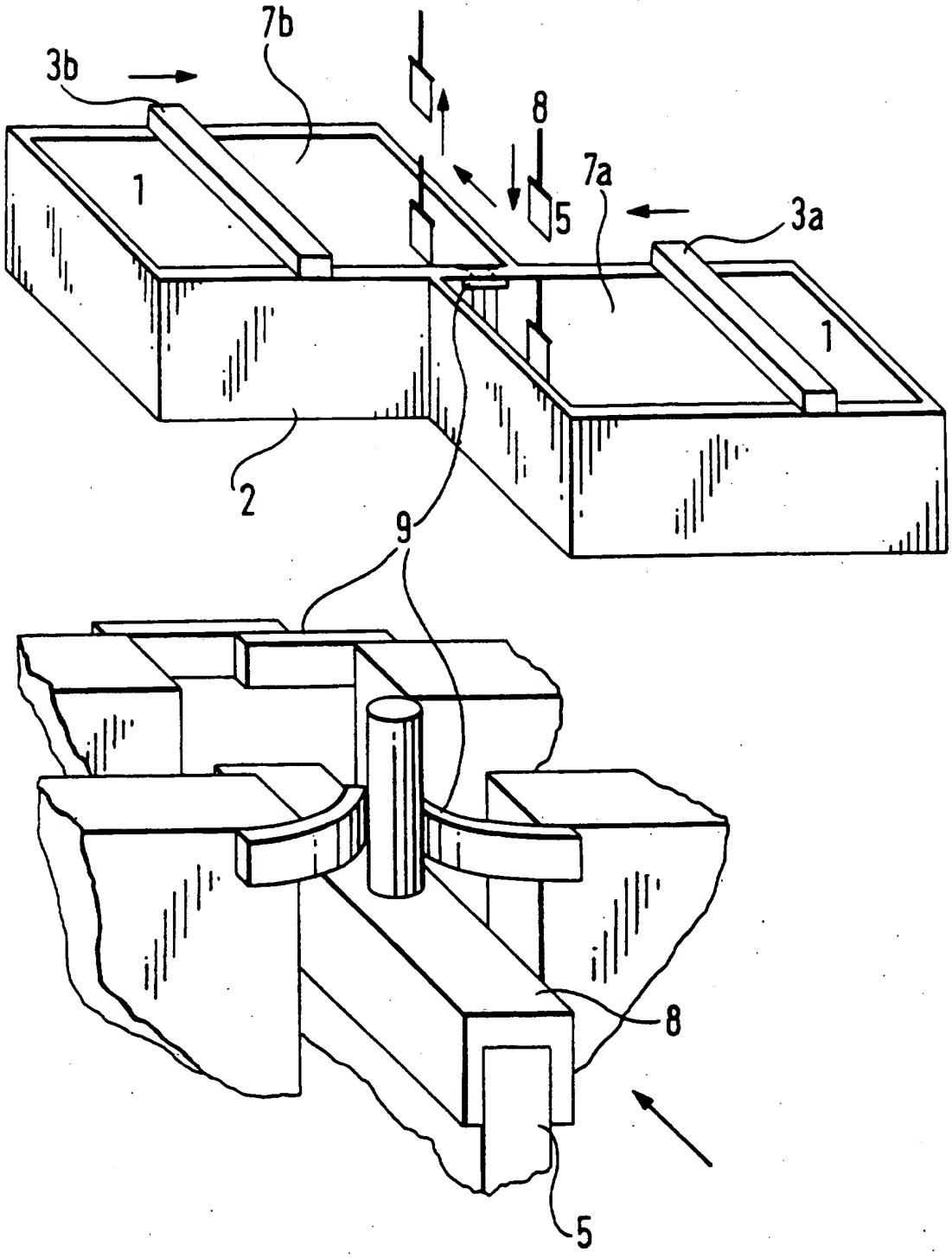


FIG. 10(f)

FIG. 11



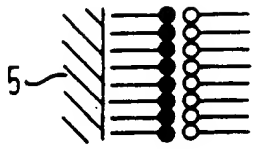


FIG. 12(j)

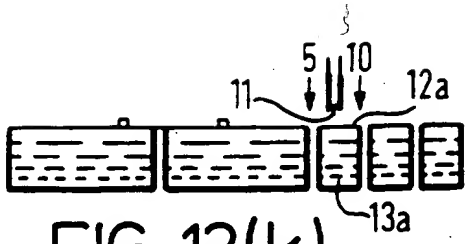


FIG. 12(k)

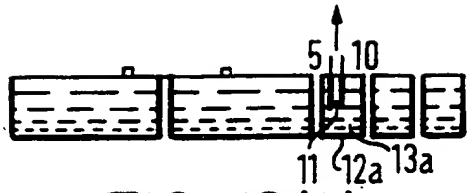


FIG. 12(l)

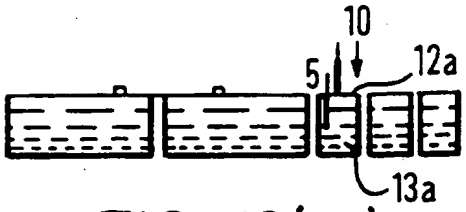


FIG. 12(m)

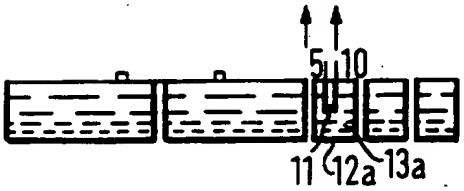


FIG. 12(n)

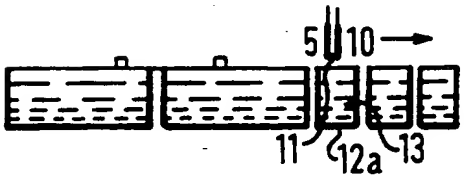


FIG. 12(o)

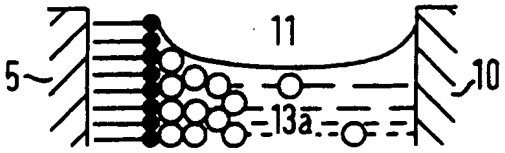


FIG. 12(p)

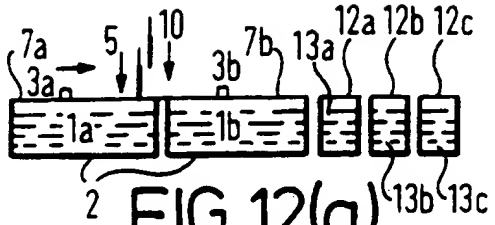


FIG. 12(a)

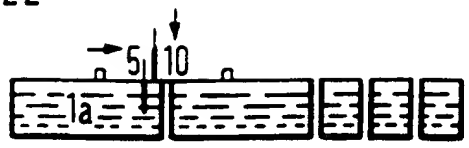


FIG. 12(b)

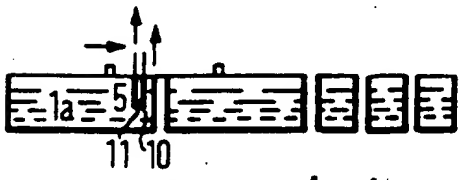


FIG. 12(c)

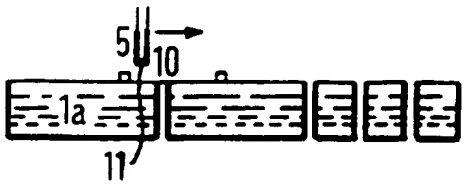


FIG. 12(d)

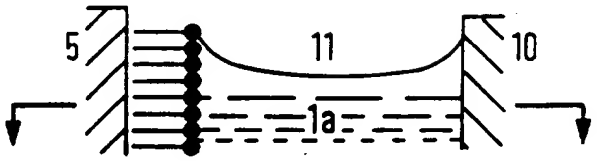


FIG. 12(e)

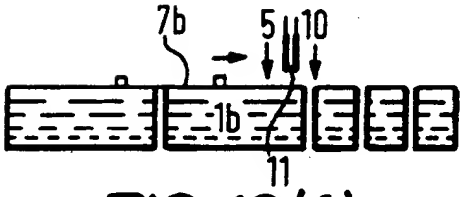


FIG. 12(f)

FIG. 12(g)

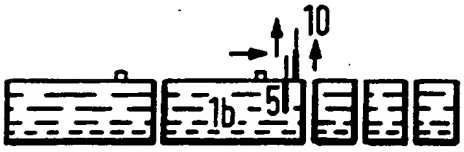


FIG. 12(h)

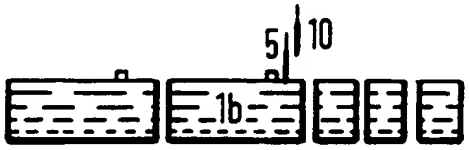


FIG. 12(i)

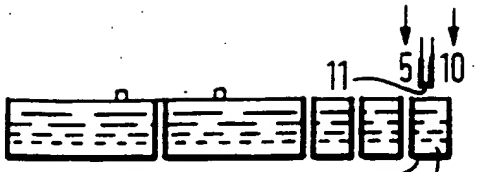


FIG. 13(a)

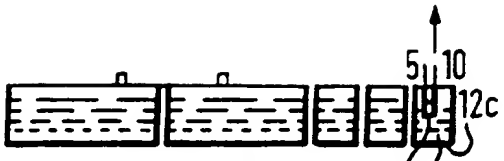


FIG. 13(b)

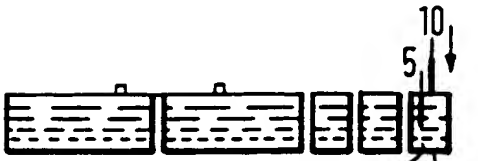


FIG. 13(c)

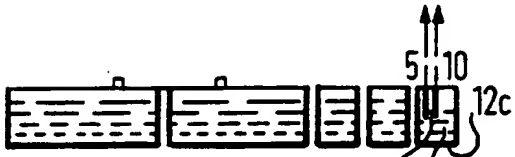


FIG. 13(d)

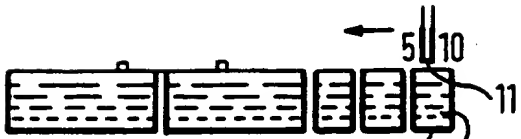


FIG. 13(e)

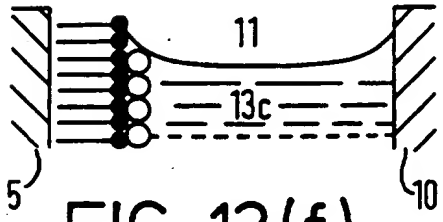


FIG. 13(f)

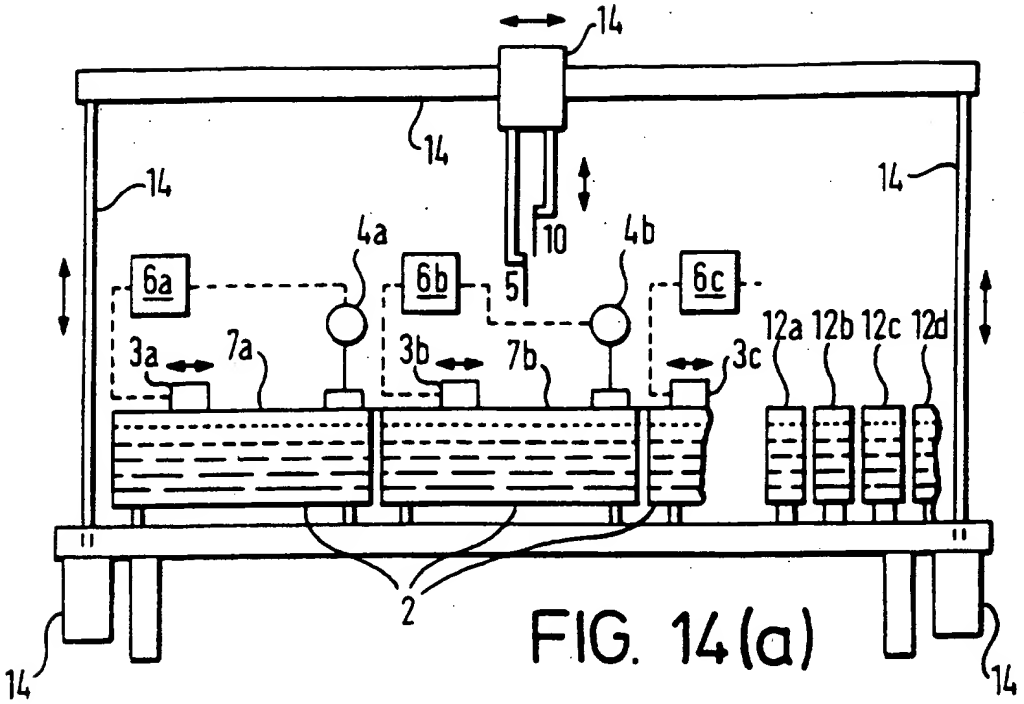


FIG. 14(a)

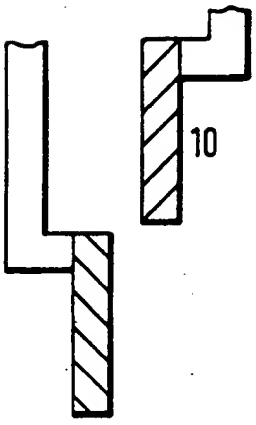


FIG. 14(b)

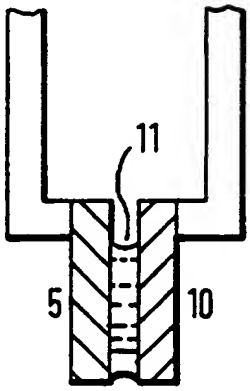


FIG. 14(c)

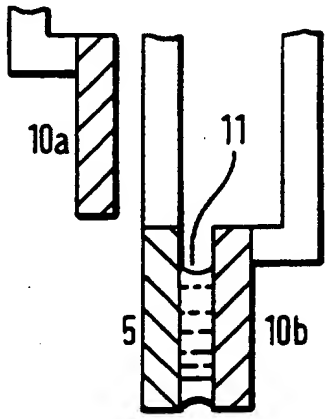
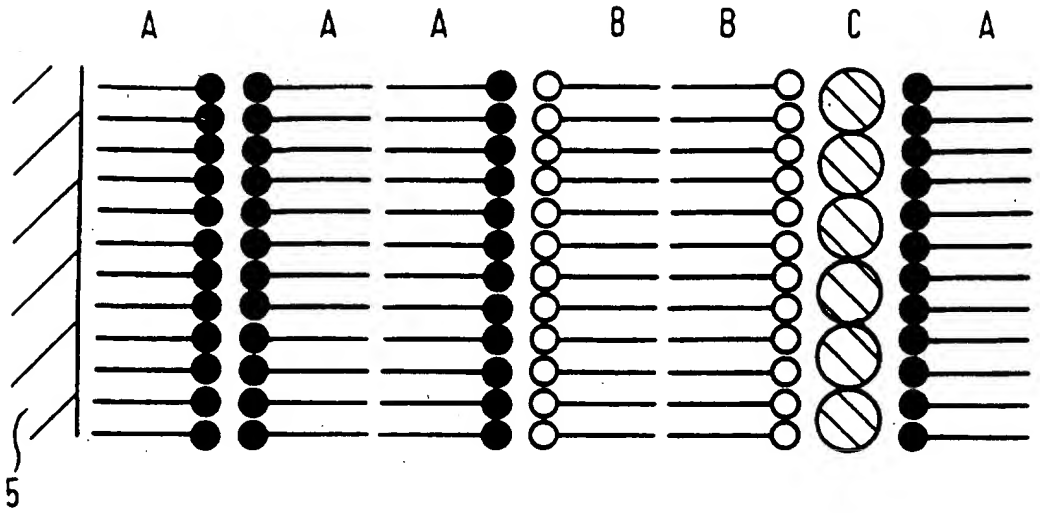


FIG. 14(d)

FIG. 15


A
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B
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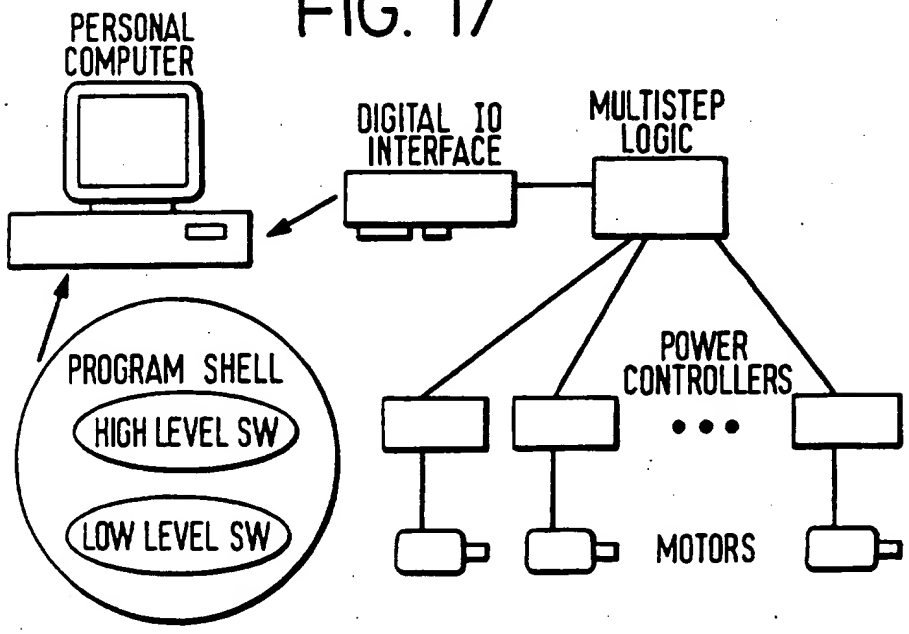
A
5

FIG. 17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LOW LEVEL SW
POWER CONTROLLERS
MOTORS

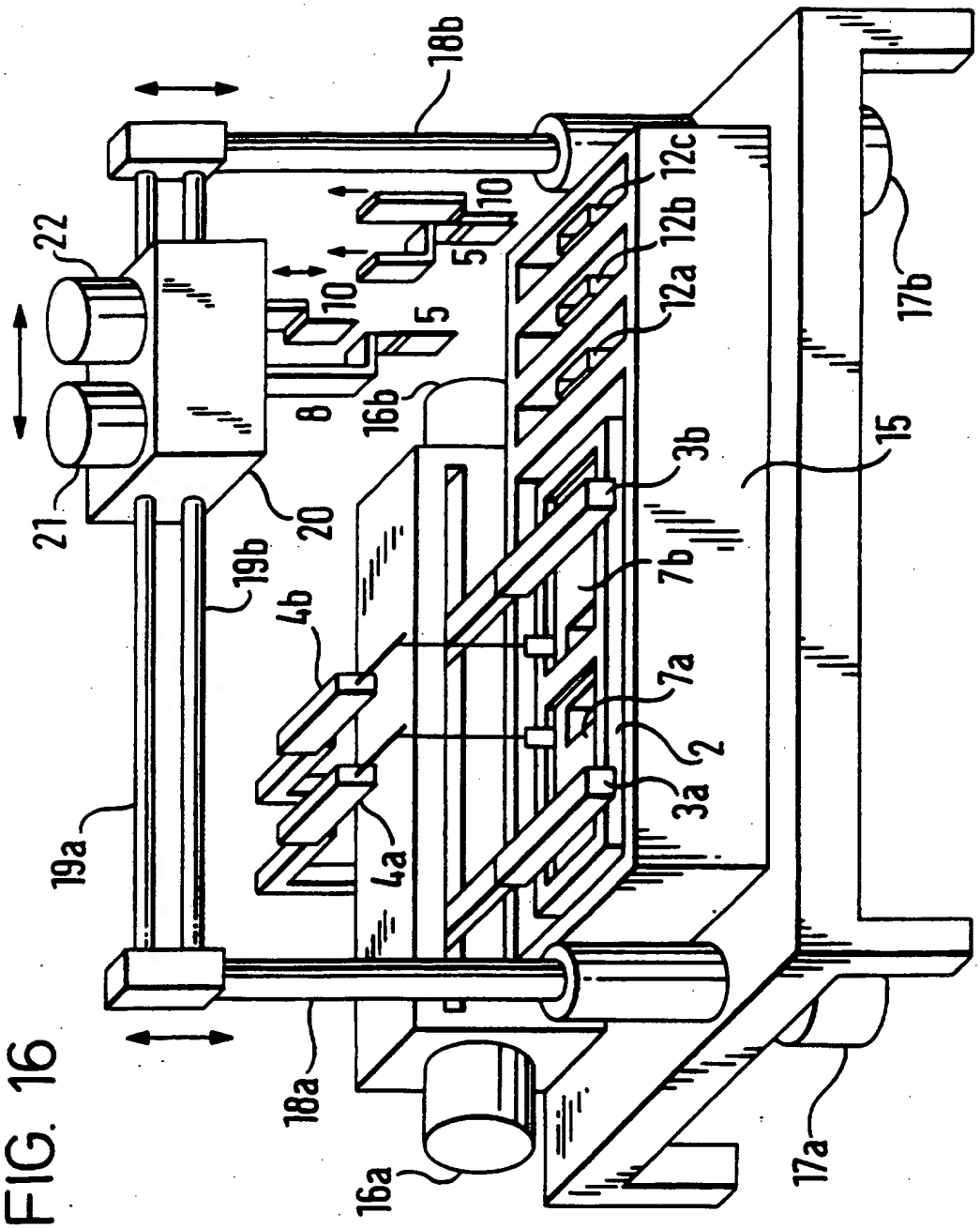
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FIG. 16

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N27/327 G01N33/543 B05D1/20 B05C3/02 B05C3/09

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N B05D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Section Ch, Week 8838 Derwent Publications Ltd., London, GB; Class B04, AN 88-268435 & JP,A,63 196 832 (NIPPON OIL SEAL IND.) , 15 August 1988 see abstract ---	
A	DATABASE WPI Section Ch, Week 9251 Derwent Publications Ltd., London, GB; Class D16, AN 92-419721 & JP,A,04 315 054 (OKI ELECTRIC IND CO LTD) , 6 November 1992 see abstract ---	
A	US,A,4 735 906 (G. J. BASTIAANS) 5 April 1988 ---	
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 November 1995	24. 11. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (- 31-70) 340-2040, Tx. 31 651 epo nl, Fax (- 31-70) 340-3016	Cartagena y Abella,P

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,1 119 126 (COMMISSARIAT A L'ENERGIE ATOMIQUE) 19 September 1984 cited in the application see the whole document	16-24
P,X	SENSORS AND ACTUATORS B, vol. 24-25, no. 1-3, March 1995 pages 121-128, C. NICOLINI ET AL. 'High-sensitivity biosensor based on LB technology and on nanogravimetry' see the whole document	1-24
P,A	ANALYTICAL CHEMISTRY, vol. 67, no. 4, 15 February 1995 pages 770-774, T. HOSHI ET AL. 'Controlled deposition of Glucose oxidase on Platinum electrode based on an avidin/biotin system for the regulation of output current of Glucose sensors.'	
P,A	WO,A,95 14962 (TECHNOBIOCHIP) 1 June 1995	
P,A	LANGMUIR, vol. 11, no. 7, July 1995 pages 2719-2715, F. ANTOLINI ET AL. 'Heat stable Langmuir-Blodgett film of Glutathione-S-Transferase.'	

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4735906	05-04-88	NONE	
EP-A-1119126		NONE	
WO-A-9514962	01-06-95	AU-B- 1075395	13-06-95